

# Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite *Leishmania tarentolae*

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**Functional cloning led to the isolation of a novel methotrexate (MTX) resistance gene in the protozoan parasite *Leishmania*. The gene corresponds to *orfG*, an open reading frame (ORF) of the LD1/CD1 genomic locus that is frequently amplified in several *Leishmania* stocks. A functional ORF G–green fluorescence protein fusion was localized to the plasma membrane. Transport studies indicated that ORF G is a high affinity bipterin transporter. ORF G also transports folic acid, with a lower affinity, but does not transport the drug analog MTX. Disruption of both alleles of *orfG* led to a mutant strain that became hypersensitive to MTX and had no measurable bipterin transport. *Leishmania tarentolae* MTX-resistant cells without their high affinity folate transporters have a rearranged *orfG* gene and increased *orfG* RNA levels. Overexpression of *orfG* leads to increased bipterin uptake and, in folate-rich medium, to increased folate uptake. MTX-resistant cells compensate for mutations in their high affinity folate/MTX transporter by overexpressing ORF G, which increases the uptake of pterins and selectively increases the uptake of folic acid, but not MTX.**

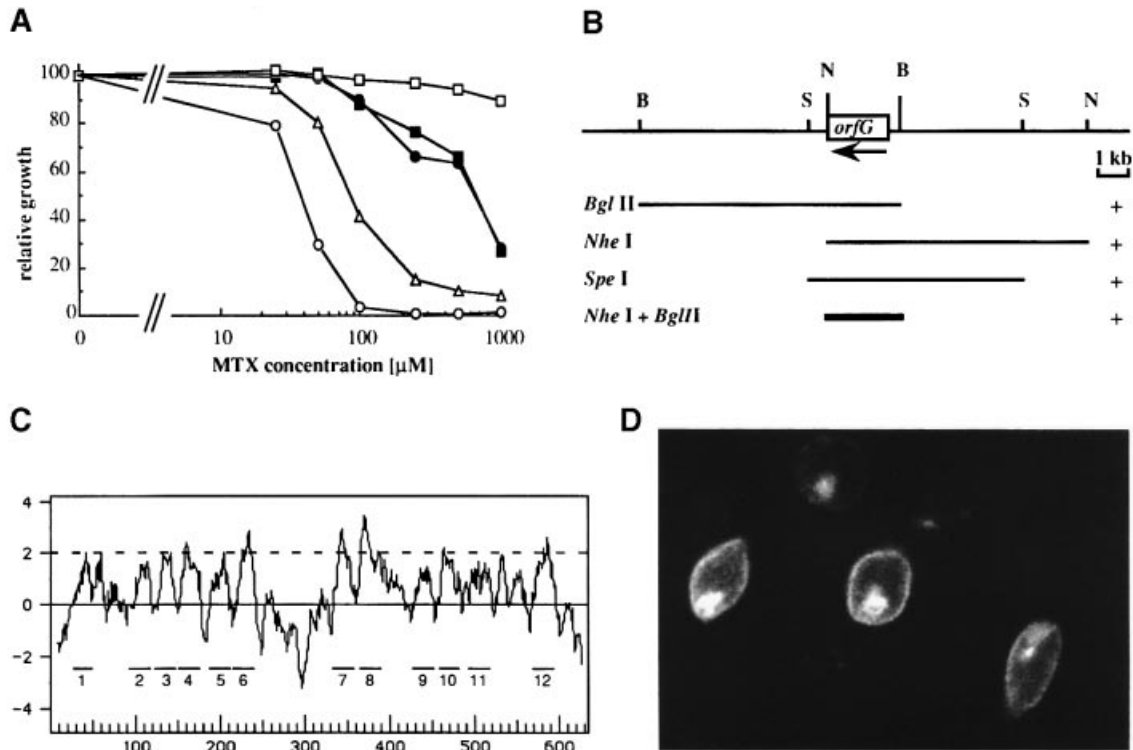
**Keywords:** bipterin/drug resistance/folate transport/*Leishmania*/methotrexate

## Introduction

Antifolates are inhibitors of the enzyme dihydrofolate reductase (DHFR), which supplies the cell with reduced folates which are essential cofactors used in many one-carbon donor reactions (Schweitzer *et al.*, 1990; Kamen, 1997). Folates are made of three building blocks: a pterin moiety which is conjugated to para amino benzoic acid by dihydropteroate synthase (DHPS) and a glutamic acid which is conjugated to dihydropteroate by dihydrofolate synthase to produce dihydrofolate. Dihydrofolate is reduced to tetrahydrofolate by DHFR. Because the DHFR proteins of different organisms share little homology, this enzyme proved to be a valuable target for chemotherapeutic drugs. Various antifolates have been successfully used as anticancer drugs (methotrexate) or in the treatment of bacterial (trimethoprim) or of parasitic infections such as malaria or toxoplasmosis (pyrimethamine) (Schweitzer *et al.*, 1990).

No successful antifolate chemotherapy has yet been established against infections with the protozoan parasite *Leishmania*. Nevertheless, many distinct features in the folate metabolism of this organism have been identified so far, which could prove useful therapeutic targets. By using methotrexate (MTX) as a model antifolate drug, several different resistance mechanisms were identified. Whereas some of them were similar to mechanisms found in cancer cells or bacteria, others turned out to be novel (Borst and Ouellette, 1995; Nare *et al.*, 1997). Gene amplification as part of extrachromosomal elements is commonly seen in response to drug selection in *Leishmania* (Beverley, 1991; Papadopoulou *et al.*, 1998). Amplification of the *dhfr-ts* gene encoding the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) leads to overexpression of the main MTX target enzyme and has been observed in *L.major* in response to drug selection (Coderre *et al.*, 1983; Ellenberger and Beverley, 1987b). In one case, a combination of overexpression with a point mutation within the *L.major* DHFR-TS was reported which resulted in a largely increased resistance level (Arrebola *et al.*, 1994). Another locus that is often amplified in several *Leishmania* species selected for MTX resistance encodes for PTR1 (pterin reductase), an enzyme belonging to the family of short chain dehydrogenase/reductases (Callahan and Beverley, 1992; Papadopoulou *et al.*, 1992). PTR1 is capable of reducing fully or partially oxidized pterins or folates (Bello *et al.*, 1994; Wang *et al.*, 1997). It is believed that overexpression of this enzyme confers MTX resistance by supplying the cell with a sufficient amount of reduced folates, thus by-passing the need for DHFR.

Besides the amplification of *ptr1* and *dhfr-ts* genes, reduction of the drug uptake is the second main pathway by which *Leishmania* resist antifolates. *Leishmania* have long been believed to be auxotrophic for folates, and during *in vitro* growth the cells rely mainly on uptake of folates for growth. One common high affinity transporter for folate and MTX has been identified in *Leishmania* and related parasites and mutations within this gene lead to antifolate resistance (Dewes *et al.*, 1986; Ellenberger and Beverley, 1987b; Kaur *et al.*, 1988; Papadopoulou *et al.*, 1993). These mutations are associated with a large variety of transport phenotypes ranging from a 2-fold decrease in folate/MTX transport to uptake levels below the detection limit. Mutant strains with no apparent measurable folate uptake are able to thrive under laboratory growth conditions. This suggests that *Leishmania* must be capable of *de novo* folate synthesis or that folates have alternative routes of entry. The conversion of radiolabeled bipterin (one of the building blocks of folic acid) into reduced folates has been demonstrated in *L.donovani* (Beck and Ullman, 1991) and is consistent with *de novo* synthesis. The exact mechanism of this conversion is unknown, but



**Fig. 1.** Functional cloning of a novel *Leishmania* MTX resistance gene. (A) Profile of MTX resistance of TarII WT ( $\circ$ ) and transfected with *dhfr-ts* ( $\triangle$ ), cMM4 ( $\blacktriangle$ ), the 2.3 kb *Bgl*III-*Nhe*I fragment of cMM4 ( $\bullet$ ) and *ptr1* ( $\square$ ). The cells were grown in SDM 79 medium supplemented with 5% heat-inactivated FBS. (B) Partial physical map of the *orfG* region of *L.mexicana*. Below the map, the restriction fragments associated with MTX resistance when transfected on a multicopy expression vector are depicted. B, *Bgl*III; N, *Nhe*I; S, *Spe*I. (C) Hydrophobicity plot of ORF G of *L.mexicana* (Kyte and Doolittle, 1982). Putative transmembrane segments are underlined and numbered. The sequence of the *L.mexicana orfG* gene can be found under the DDBJ/EMBL/GenBank accession No. AF078929. (D) Confocal laser scanning microscopy of *L.tarentolae* overexpressing the ORF G-GFP fusion protein, showing the membrane location of ORF G.

seems to differ from the conventional route via DHPS since incorporation of radiolabeled *p*-amino benzoic acid could not be detected in *L.major* (Kovacs *et al.*, 1989), and several DHPS inhibitors are not active against *Leishmania* (Peixoto and Beverley, 1987; Kaur *et al.*, 1988).

By transfecting a *L.tarentolae* gene bank into wild-type parasites and selecting for MTX resistance, we isolated a novel resistance gene coding for a high affinity membrane bipterin transporter, which also has low affinity for folic acid transport but does not transport MTX. *Leishmania tarentolae* cells resisting MTX by mutations in their common high affinity folate/MTX transporter showed an increase in the activity of their bipterin transporter.

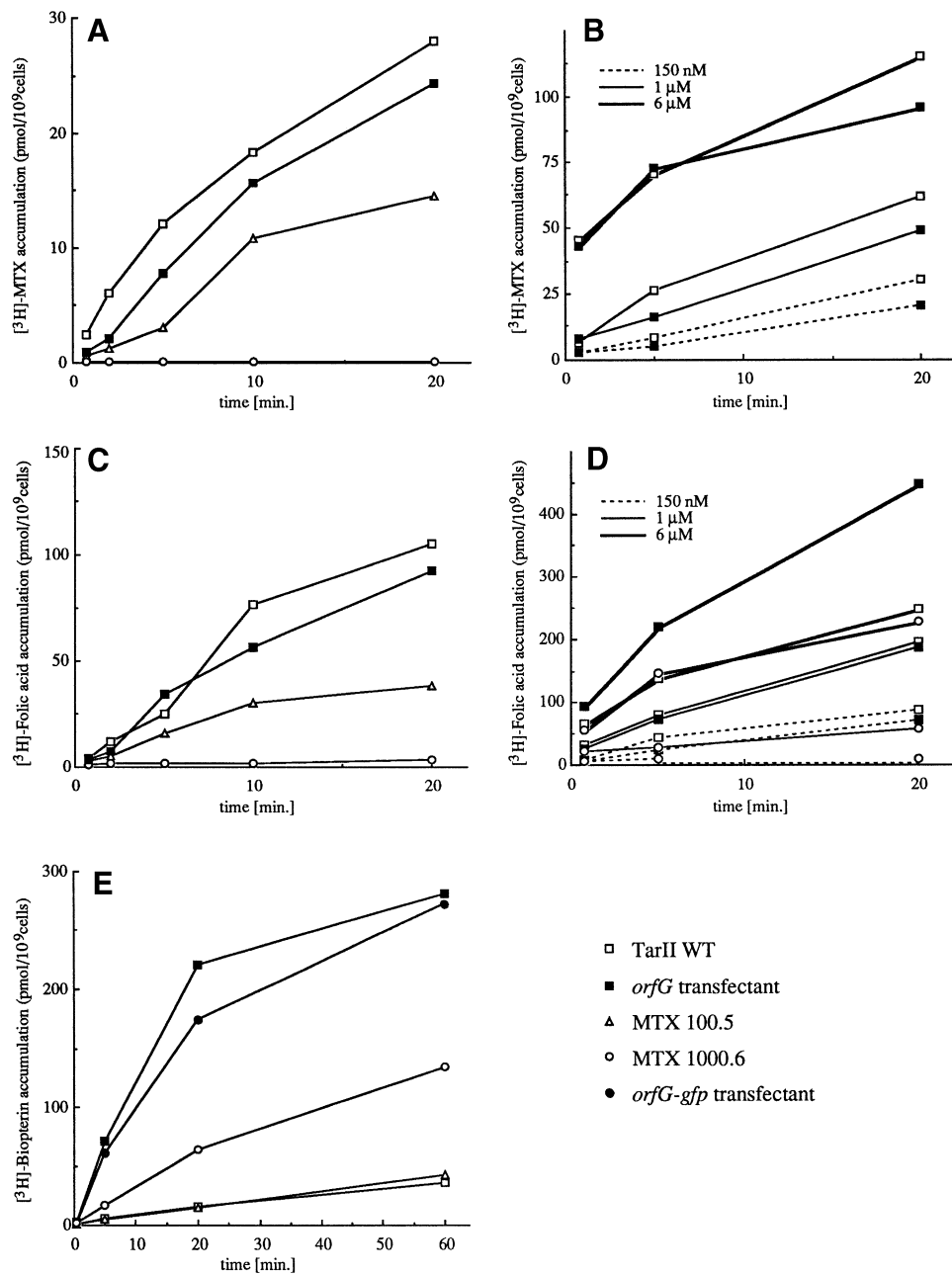
## Results

### Functional cloning of the novel MTX resistance gene *orfG*

Drug resistance genes in *Leishmania* are usually isolated by analyzing mutants selected for resistance by increasing drug concentrations (Borst and Ouellette, 1995). In order to isolate new resistance genes, we used functional cloning which was initially set up to study genes involved in lipophosphoglycan biosynthesis (Descoteaux *et al.*, 1994). Wild-type *L.tarentolae* cells were transfected with a genomic cosmid bank and plated on MTX-containing plates (see Materials and methods). An identical cosmid called cMM4 was found in five of the transfectants obtained by functional cloning. Retransfection of the

cosmid cMM4 into *L.tarentolae* TarII wild type (WT) restored the MTX resistance and this transfectant showed an increase of its  $EC_{50}$  by  $\sim$ 10-fold when compared with wild-type cells (Figure 1A). The level of resistance conferred by cMM4 differs from *L.tarentolae* cells transfected with cosmids containing either the *dhfr-ts* or *ptr1* gene (Figure 1A), suggesting the presence of a novel MTX resistance gene on cosmid cMM4. The novelty of the resistance gene was confirmed by hybridization experiments since neither a *ptr1* nor a *dhfr-ts* probe hybridized to cMM4 (not shown). The cosmid cMM4 was digested with either *Bgl*III, *Nhe*I or *Spe*I, and subcloned into the *Leishmania* expression vector pSPY-hyg (Papadopoulou *et al.*, 1994b). After transfection in TarII WT, three different restriction fragments, a 6 kb *Bgl*III, a 6.8 kb *Spe*I and an 8.5 kb *Nhe*I fragment were associated with MTX resistance. A 2.3 kb *Bgl*III-*Nhe*I fragment was the smallest segment common to all three fragments (Figure 1B). Transfection of this fragment conferred a similar level of MTX resistance as the original cosmid cMM4 (Figure 1A).

DNA sequence analysis of the 2.3 kb *Bgl*III-*Nhe*I fragment (DDBJ/EMBL/GenBank accession No. AF078929) revealed an open reading frame (ORF) of 1893 bp with the TAG stop codon being part of the *Nhe*I site used for the subcloning of the gene. The ORF shared 88% identity with ORF G of *L.donovani*. ORF G was first described as part of LD1/CD1 amplicons spontaneously occurring in various *Leishmania* species (Myler *et al.*, 1994). ORF G also shares considerable homology with

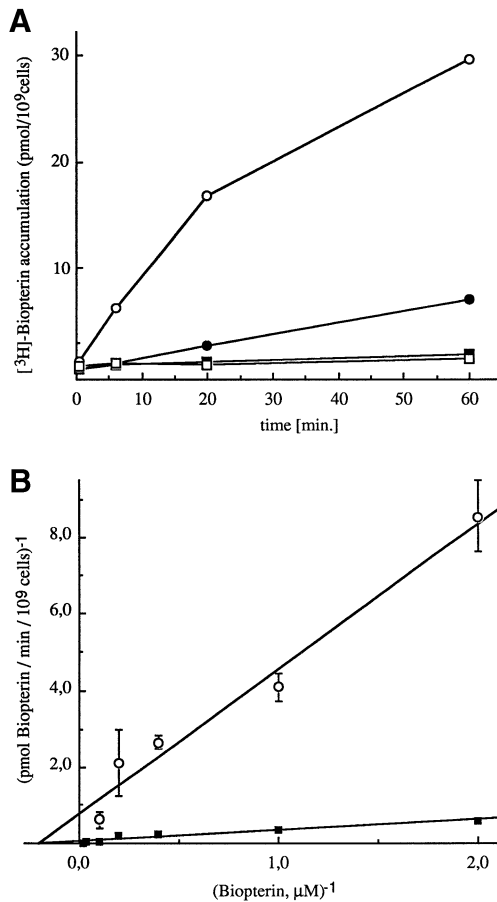


**Fig. 2.** Accumulation of radiolabeled pteridines in *Leishmania* wild-type and MTX-resistant cells. The transport studies with TarII WT (□), TarII transfected with *orfG* (■), MTX 100.5 (△) and MTX 1000.6 (○) were carried out as described in Materials and methods. (A) [<sup>3</sup>H]MTX accumulation using 150 nM of the drug. (B) [<sup>3</sup>H]MTX accumulation using varying concentrations of the drug. (C) [<sup>3</sup>H]folate accumulation using 150 nM of substrate. (D) [<sup>3</sup>H]folate accumulation using varying concentrations of the substrate. (E) [<sup>3</sup>H]biopterin accumulation; TarII transfected with an *orfG*-GFP fusion (●). Transport experiments with each individual cell line have been done at least three times and similar results were obtained consistently.

ESAG10 of *Trypanosoma brucei*, a protein with unknown function encoded by an expression site-associated gene (Gottesdiener, 1994). The *orfG* gene was identified as a MTX resistance gene by functional cloning. It is possible that during selection we may have selected for a point mutation within the gene which is responsible for the observed resistance phenotype. To address this possibility, the *orfG* genes of *L.mexicana*, *L.donovani* and *L.tarentolae* were cloned in a *Leishmania* expression vector. Upon transfection, all these genes produced a similar level of MTX resistance as observed with the original cosmid cMM4 (not shown). These experiments indicated that

the wild-type *orfG* genes from at least three different *Leishmania* species are able to confer antifolate resistance.

Hydrophobicity analysis of ORFG suggested the presence of 12 putative transmembrane segments (Figure 1C). Most of these transmembrane domains contain one or more hydrophilic amino acid residues that are predicted to form amphiphilic  $\alpha$ -helices or  $\beta$ -strands, a structure that is typical for type IV integral membrane proteins (Singer, 1990). It has been suggested that members of this class of membrane proteins act as aqueous channels through the membrane and mediate specific transport of small hydrophilic molecules. To confirm the membrane

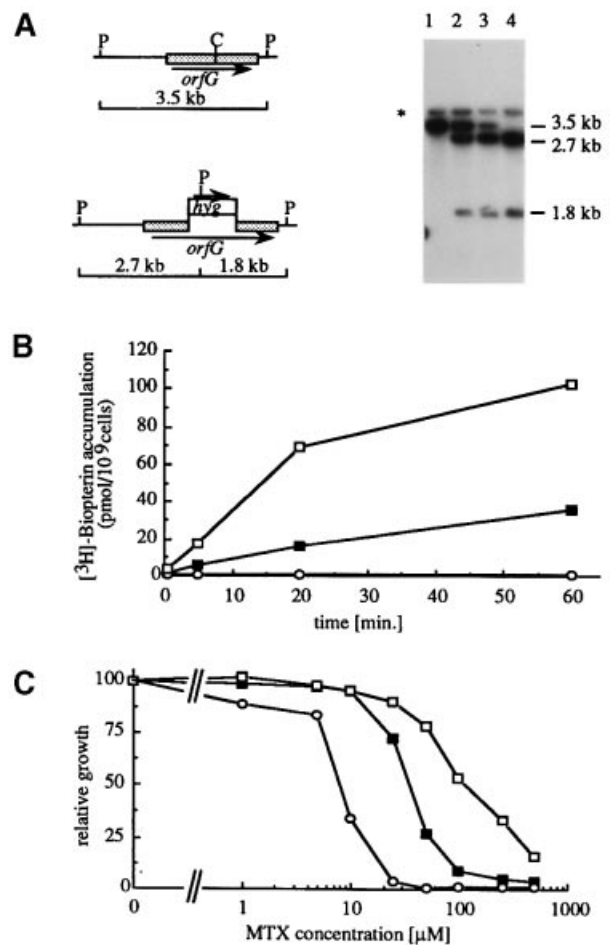


**Fig. 3.** Characterization of the bioplerin transporter ORF G. (A) Bioplerin transport (500 nM) in *L.tarentolae* wild-type cells (○) or incubated on ice (□), or in the presence of 20 mM sodium azide (●) or of 5 mM 2,4-dinitrophenol (■). (B) Lineweaver–Burk analysis of bioplerin transport in TarII WT cells (○) or Tar II cells transfected with *orfG* (■). Apparent  $K_m$  and  $V_{max}$  values found in Table I were determined from the intercepts of the  $x$ - and  $y$ -axes.

**Table I.** Biochemical characteristics of pteridine transport in *L.tarentolae*

Cells	Bioplerin		Folate	
	$K_m$ (μM)	$V_{max}$ (pmol/min/ $10^9$ cells)	$K_m$ (μM)	$V_{max}$ (pmol/min/ $10^9$ cells)
Tar II wild type	4.9	1.28	0.26	7.6
<i>orfG</i> transfectant	4.7	17.1	0.45	8.1

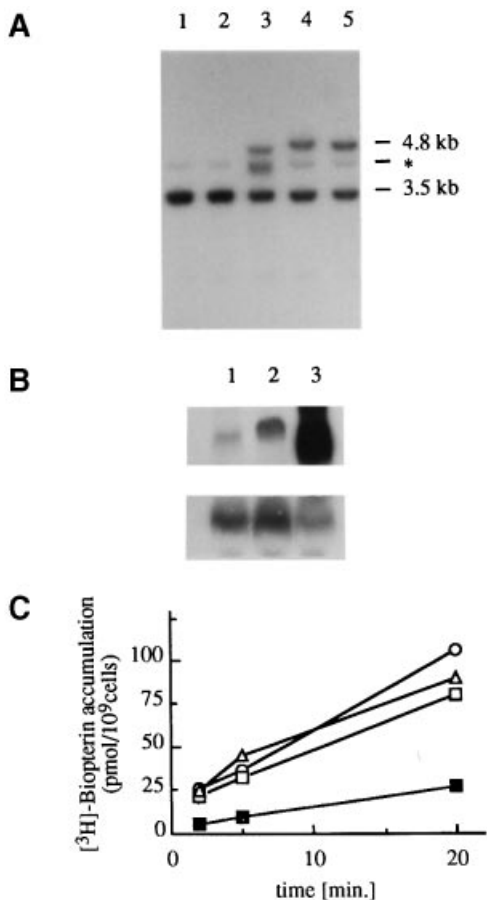
location of ORF G, we constructed an ORF G–green fluorescent protein (ORF G–GFP) fusion. This fusion has the same activity as the intact ORF G since it confers the same level of MTX resistance (not shown) and it has the same pteridine transport properties (Figure 2). This suggests that the cellular location of the overexpressed ORF G–GFP is similar to that of the overexpressed ORF G. The localization of the ORF G–GFP fusion was studied by confocal microscopy (Figure 1D). Uniform staining of the plasma membrane was observed. In addition, the fusion protein was also detected within an intracellular compartment at the base of the flagellum that probably corresponds to the flagellar pocket.



**Fig. 4.** Analysis of a *L.tarentolae orfG*-null mutant. (A) Southern blot analysis of an *orfG*-null mutant. Total DNA was digested with *Pst*I and hybridized to an intragenic *orfG* probe. Lane 1, TarII WT; lane 2, total population of *orfGYhygro* mutants; lane 3, *orfGYhygro* mutants selected with high levels of hygromycin B; lane 4, *IIforfGYhygro.2*, an *orfG*-null mutant. An *orfG* homologous gene is marked by an asterisk. A partial physical map of the *orfG* region of *L.tarentolae* wild-type and the *orfG*-null mutant is shown. Fragments obtained after *Pst*I digestion are depicted below the map. P, *Pst*I; C, *Csp45I*. (B) Measurement of bioplerin accumulation. TarII WT (■), *IIforfGYhygro.2* (○), *IIforfGYhygro.2* transfected with *orfG* (□). (C) MTX resistance of TarII WT (■), *IIforfGYhygro.2* (○) and *IIforfGYhygro.2* transfected with *orfG* (□).

#### Pteridine transport properties of MTX-resistant *L.tarentolae* and of an *orfG* transfectant

Computer analysis of ORF G suggested that it can specifically transport hydrophilic molecules. One possible way to increase the level of resistance would be an accelerated extrusion of the drug outside the cell (Borst and Ouellette, 1995). To test this possibility we measured the steady-state accumulation of [<sup>3</sup>H]MTX in wild-type and MTX mutant cells as well as in an *orfG* transfectant (Figure 2A). We have previously described two classes of *L.tarentolae* MTX/folate transporter mutants with a decreased uptake of 50 and >95%, respectively (Papadopoulou *et al.*, 1993). As reported in the past, the mutant MTX 100.5 showed a 2-fold decrease in uptake while no MTX uptake could be measured in mutant MTX 1000.6 (Figure 2A). The accumulation of folic acid in wild-type and MTX mutant cells was very similar to the kinetics of MTX uptake although some folate uptake could



**Fig. 5.** Overexpression of ORF G in MTX-transport mutants. (A) Southern blot analysis of total DNA of *L.tarentolae* TarII WT and MTX-transport mutants: total DNA was digested with *Pst*I and hybridized with an intragenic *orfG* probe. Lane 1, TarII WT; lane 2, MTX 100.5; lane 3, MTX 1000.3; lane 4, MTX 1000.6; lane 5, MTX 1000.7. (B) Analysis of *orfG* RNA by Northern blot. Total RNA was hybridized to an intragenic *orfG* fragment of *L.mexicana* and re-hybridized to an  $\alpha$ -tubulin gene probe to monitor the amount of RNA layered in each lane. Lane 1, TarII WT; lane 2, MTX 1000.6; lane 3, TarII *orfG* transfectant. (C) Measurement of biopterin accumulation. TarII WT (■), MTX 1000.3 (○), MTX 1000.6 (□), MTX 1000.7 (△).

be detected in MTX1000.6 (Figure 2C), further suggesting that *Leishmania* has a common folate/MTX transporter (Ellenberger and Beverley, 1987b). Overexpression of *orfG* led to no significant change in MTX accumulation compared with wild-type levels, indicating no involvement of ORF G in MTX export (Figure 2B). The sensitivity of *Leishmania* to antifolate drugs is heavily influenced by the concentration of exogenous folates (Beverley, 1991; Papadopoulou and Ouellette, 1993). Therefore, a selective increase of folate import compared with MTX uptake is another possible mechanism by which the putative transmembrane transporter ORF G could confer MTX resistance. Under standard conditions, the *orfG* transfectant showed no significant increase in folate transport compared with wild-type cells (Figure 2C). At higher concentrations of folate, however, ORF G effectively transported folic acid (Figure 2D). ORF G seems therefore to correspond to a low affinity folate transporter that can discriminate between MTX and folic acid (Figure 2B and D).

We also tested the possible involvement of ORF G in the

transport of pterins using radioactive biopterin. Biopterin enables growth of *Leishmania* cells in a defined folate-deficient medium (Kaur *et al.*, 1988; Beck and Ullman, 1990; Bello *et al.*, 1994; Papadopoulou *et al.*, 1994a). The uptake of biopterin was shown to be transport mediated (Beck and Ullman, 1990), and radiolabeled biopterin can be incorporated into reduced folate (Beck and Ullman, 1991). Wild-type *L.tarentolae* cells were shown to accumulate [<sup>3</sup>H]biopterin in a time-dependent manner (Figure 2E). Using standard conditions, under which no increase in folate transport could be detected (Figure 2C), the accumulation of [<sup>3</sup>H]biopterin was increased 10-fold in the *orfG* transfectant compared with wild-type cells and a similar increase was observed with the ORF G–GFP fusion (Figure 2E). MTX 100.5, a cell line with a 2-fold reduced MTX/folate uptake, accumulated biopterin at the same rate as wild-type cells. Interestingly, the cell line MTX 1000.6, without any detectable activity of its high affinity MTX/folate transporter, showed a biopterin accumulation that was several times greater than the wild-type level (Figure 2E).

The uptake of biopterin in *L.tarentolae* is probably mediated by an active transport mechanism as no accumulation could be measured when the cells were incubated on ice (Figure 3A). This was substantiated by the lack of biopterin accumulation in cells treated with the metabolic inhibitors sodium azide (20 mM) and 2,4-dinitrophenol (5 mM) (Figure 3A). Similar concentrations of inhibitors were shown to inhibit active folate uptake (Ellenberger and Beverley, 1987a) and active efflux of arsenite (Dey *et al.*, 1994) in *Leishmania*. To characterize further the biopterin transport properties of ORF G, we measured the rate of uptake of biopterin in a wild-type cell and in an *orfG* transfectant while varying biopterin concentration. Wild-type cells exhibit uptake of biopterin with high affinity and an apparent  $K_m$  of 4.9  $\mu$ M (Figure 3B; Table I). A similarly high affinity biopterin with an apparent  $K_m$  of 4.7  $\mu$ M was observed for the *orfG* transfectant while its  $V_{max}$  was increased by >10-fold (Figure 3B; Table I). The increase in the rate of uptake (Table I) correlates well with the levels of the steady-state accumulation of biopterin observed in wild-type cells and *orfG* transfectant cells (Figure 2E).

Wild-type *Leishmania* cells also have a high affinity folate transporter with a  $K_m$  value of 0.7  $\mu$ M for *L.major* (Ellenberger and Beverley, 1987a) and 0.23  $\mu$ M in *L.donovani* (Kaur *et al.*, 1988). We have performed Lineweaver–Burk analysis of folate transport in *L.tarentolae* and found an apparent  $K_m$  of 0.26  $\mu$ M in wild-type cells (Table I). Similar kinetic parameters were observed for folate transport in *L.tarentolae orfG* transfectant (Table I), indicating that ORF G is not the high affinity folate transporter. Nonetheless, the *L.tarentolae* ORF G can transport folic acid (Figure 2D), at least when folate concentration is at 6  $\mu$ M. Attempts to characterize the ORF G-mediated folate transport activity in more detail were unsuccessful mainly for technical reasons (see Materials and methods). Although a precise  $K_m$  value could not be determined for folate transport mediated by ORF G, results indicated clearly that ORF G contributes only marginally, at least at low folate concentration, to folate transport (Figure 2D; Table I). Overall, our transport studies indicated that ORF G is a high affinity active biopterin

transporter and a low affinity folate transporter, but does not transport the drug analog MTX (Figure 2B and D).

### Phenotype of the *L.tarentolae orfG* knock-out mutant

To characterize further the role of ORF G in the pteridine metabolism of *Leishmania*, an *orfG*-null mutant was generated. The first allele was disrupted using an hygromycin phosphotransferase (*hyg*) expression cassette (Papadopoulou *et al.*, 1994b). Southern blot analysis indicated that *orfG* is part of a 3.5 kb *PstI* fragment in wild-type cells (Figure 4A). After longer exposure we could detect several weaker fragments hybridizing to an *orfG* probe. One of these, which was not affected by the construction of *orfG* mutants, is visible in Figure 4A (marked by an asterisk). Analysis of the hygromycin-resistant cell pool showed the appearance of two additional fragments of 2.7 and 1.8 kb, which is consistent with the introduction of an additional *PstI* site within the *hyg* marker into *orfG* (Figure 4A). *Leishmania* is diploid and this makes it necessary to inactivate the second allele which can conveniently be done by loss of heterozygosity (Gueiros-Filho and Beverley, 1996). By increasing the selection pressure with hygromycin B, we observed an increase of the strength of the hybridization signal of the mutant fragments of 2.7 and 1.8 kb over the wild-type 3.5 kb fragment (Figure 4A, lane 3), suggesting that we have enriched for double disruptants within the cell pool. Cloning of this culture led to the isolation of the *orfG*-null mutant *IIorfGYhygro.2*, in which both alleles of *orfG* were disrupted by the *hyg* resistance marker (Figure 4A, lane 4).

No biopterin accumulation could be measured in *IIorfGYhygro.2* (Figure 4B), but by introducing an expression vector carrying the *orfG* wild-type gene into the *orfG* null mutant we were able to revert the transport phenotype of the mutant (Figure 4B). Inactivation of the high affinity biopterin transporter ORF G decreases the EC<sub>50</sub> to MTX by ~4-fold compared with wild-type cells (Figure 4C). By overexpressing *orfG* on a plasmid in the *orfG* null mutant, we were able to reverse the hypersensitivity to MTX and produce a level of resistance which is close to that we have observed in wild-type cells overexpressing *orfG* (Figures 1A and 4C). An *orfG* null mutant of *L.tarentolae* is viable in culture and shows only a very small delay in its growth rate compared with wild-type cells. ORF G is therefore not essential for growth of *L.tarentolae* in culture medium, although it seems to be the only high affinity transporter for biopterin.

### Overexpression of ORF G in folate transport mutants

The folate transport-deficient mutant MTX 1000.6 accumulates biopterin at a rate ~3–5 times higher compared with wild-type cells (Figure 2C). As gene amplification is a common mechanism by which *Leishmania* survive drug challenge (Beverley, 1991; Papadopoulou *et al.*, 1998), we tested whether amplification of *orfG* was responsible for increased biopterin transport in *L.tarentolae* MTX 1000.6 mutants. A 3.5 kb *PstI* fragment is recognized by an *orfG* probe in wild-type cells (Figure 5A, lane 1). The same probe also recognizes one copy of the *orfG* gene family (marked with an asterisk in Figure 5A). Gene amplification or DNA rearrangements could neither be

detected in the mutant MTX 100.5 (Figure 5A, lane 2), nor in MTX 1000.4 or MTX 1000.5 (not shown), mutants in which folate/MTX transport was only reduced by 2-fold (Papadopoulou *et al.*, 1993) (Figure 2A and C). However, novel non-amplified *PstI* fragments at a size of ~4.8 kb hybridized to an *orfG* probe in mutants MTX 1000.6, but also in mutants MTX 1000.3 and MTX 1000.7 (Figure 5A, lanes 3–5). No measurable high affinity MTX/folate transport can be detected in the latter three mutants (Papadopoulou *et al.*, 1993). The precise rearrangements may differ between the three mutants as the sizes of the rearranged fragments differ slightly. All three mutants showing rearrangement within the *orfG* region also demonstrated an increase in biopterin transport (Figure 5C). All mutants that resist MTX by a mutation in their high affinity MTX/folate transport are compensating by increasing the activity of the high affinity biopterin transporter.

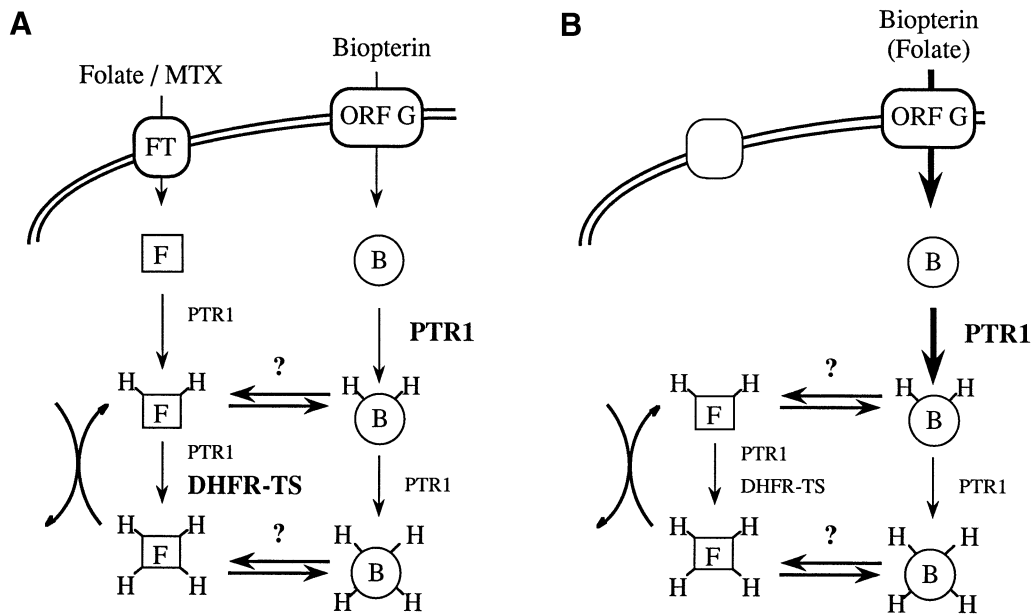
The increase in biopterin transport in the mutants was not due to *orfG* gene amplification but to an increased steady-state accumulation of *orfG* RNA by ~5-fold in MTX 1000.6 (Figure 5B, lane 2). In addition to the increased amount of RNA, we also noted that the *orfG* RNA in MTX 1000.6 (at least part of it) is larger than the wild-type RNA, which could possibly be a result of the described genomic rearrangement. The RNA of the transfectant also differs in size (Figure 5B, lane 3), but this is not surprising as only the protein-coding region of ORF G (without its own RNA maturation sequences) was cloned in a *Leishmania* expression vector. Overexpression of ORF G in MTX 1000.6 not only increases biopterin uptake but also augments uptake of folate when a high folate concentration was present in the transport assay (Figure 2D).

The increase of the steady-state *orfG* RNA level in MTX 1000.6 is commensurate with the high biopterin transport activity of this mutant. In one previous report, the RNA of *orfG* was shown to be increased following translocation of an *orfG* segment into the ribosomal locus (Lodes *et al.*, 1995). To test whether this also occurred in the mutant MTX 1000.6, the chromosomes of wild-type and MTX 1000.6 cells were separated by TAFE and hybridized to an *orfG* and a ribosomal DNA probe. The *orfG* gene is part of a 2.1 Mb chromosome while ribosomal RNA genes are part of 1.5 and 1.8 Mb chromosomes. We could detect neither any gross gene rearrangements nor translocation of *orfG* into the ribosomal locus (not shown).

## Discussion

### Isolation of a new MTX resistance gene by functional cloning

*Leishmania* often resists *in vitro* drug selection by amplifying specific portions of its genome as part of extrachromosomal elements (Beverley, 1991; Papadopoulou *et al.*, 1998). Characterization of amplicons derived from MTX-resistant *Leishmania* led to the identification of *dhfr-ts* and *prt1* genes. The presence of repeated sequences flanking the resistance genes enhanced greatly the frequency with which gene amplification events are selected in *Leishmania* (Grondin *et al.*, 1996). It is therefore likely that genes lacking such repeated flanking regions will not be amplified while selecting for stepwise increased resistance. To



**Fig. 6.** Model of the role of ORF G in MTX resistance of *Leishmania*. (A) The main route of entry of folate (and antifolates) into *Leishmania* wild-type cells is the high affinity folate transporter (FT), while pterins are taken up by the high affinity transporter ORF G. DHFR-TS reduces dihydrofolate to tetrahydrofolate. The main role of PTR1 is reduction of (bio)pterin but the enzyme can also reduce conjugated pterins. The arrows between the biopterin and folate pathways symbolize reported MTX hydrolase activity (Kaur *et al.*, 1988; Ellenberger *et al.*, 1989) and conversion of biopterin into tetrahydrofolate (Beck and Ullman, 1991). Gene amplification of *ptr1* and *dhfr-ts* leads to antifolate resistance (Borst and Ouellette, 1995; Nare *et al.*, 1997). (B) Mutations in FT confer high-level antifolate resistance but would decrease the intracellular concentration of folates. The lack of this high affinity folate uptake system is, however, compensated by overexpression of ORF G, which increases pterin uptake which presumably can be converted into folates. ORF G is also a low affinity folate transporter but does not transport MTX and this activity may also contribute to resistance, especially in cells grown under high folate concentration.

identify such genes, we introduced a *Leishmania* cosmid genomic library into wild-type cells and selected for genes which confer MTX resistance when overexpressed from a multicopy vector. The *orfG* gene was isolated in this way and constitutes a novel resistance gene. Functional cloning can therefore serve as a useful complementary approach to mutant analysis for the isolation of resistance genes. Using functional cloning, but screening for the ability of a *L.donovani* MTX-resistant mutant to thrive in folate-deficient medium supplemented with biopterin, the group of S.Beverley (Washington University, St Louis, MO) has independently isolated *orfG* and shown that it can transport biopterin (Moore and Beverley, Woods Hole Molecular Parasitology Meeting 1996, abstract 107, cited in Segovia and Ortiz, 1997).

ORF G is present in a chromosomal region called the LD1/CD1 locus (Stuart, 1991; Segovia and Ortiz, 1997). This region is present on a large 2.2 Mb *L.donovani* chromosome, but is also frequently amplified as part of linear or circular amplicons in several *Leishmania* species either cultured in the laboratory or isolated from the field. These linear or circular elements differ largely in size, ranging from 27 to >250 kb, but contain a 10 kb common region with four ORFs named ORF F, G, H and I (Myler *et al.*, 1994). The role of these ORFs is unknown although sequence analysis indicated that ORF I contains a potential ATP/GTP binding motif, while ORF G has putative transmembrane domains and is highly similar to ESAG10 (Gottesdiener, 1994), an expression site-associated gene of unknown function present in the African trypanosomes. The membrane location of ORF G was confirmed by using a fully active ORF G-GFP fusion (Figure 1D). Fluorescence was also detected in an intracellular compart-

ment at the flagellated end of the cell, which is likely to correspond to the flagellar pocket, a site where receptor-mediated endocytosis occurs (Webster and Russell, 1993). The generation of antibodies recognizing ORF G will be necessary to determine whether ORF G also localizes to that compartment in wild-type cells. The *Leishmania* integral glucose transporter Pro-1 was found to be located both in the plasma membrane and the flagellar pocket (Piper *et al.*, 1995), indicating that dual location of a membrane protein is not without precedent in *Leishmania*.

Contrary to their host, *Leishmania* and other related parasites lack a *de novo* pathway for the biosynthesis of pterins. Several studies have shown that *Leishmania* requires pteridines (folates or pterins) for growth (Trager, 1969; Peixoto and Beverley, 1987; Scott *et al.*, 1987; Kaur *et al.*, 1988) but biopterin and other unconjugated pterins can clearly support the growth of *Leishmania* in folate-deficient medium (Beck and Ullman, 1990; Bello *et al.*, 1994; Papadopoulou *et al.*, 1994a). Pterins are important in folate metabolism in *Leishmania* (Figure 6) but also have other important as yet unidentified roles (Bello *et al.*, 1994; Papadopoulou *et al.*, 1994a), including one in oxidant resistance (Nare *et al.*, 1997). The frequent amplification of LD1/CD1 in various *Leishmania* species may therefore be due to the presence of ORF G which can increase the uptake of pterins required in larger amounts under certain conditions, such as nutritional or environmental stresses, encountered throughout their life cycle. The high affinity biopterin transporter seems to be non-essential in *L.tarentolae*, as an *orfG*-null mutant with no measurable biopterin uptake (Figure 4B) could still grow in culture medium. The pterin requirements of this mutant are likely to be met by a pteridine hydrolyzing enzyme

(Figure 6), which was described in *Leishmania* and other related parasites (Oe *et al.*, 1983; Kaur *et al.*, 1988; Ellenberger *et al.*, 1989), and/or by other low affinity transporters that were not detected under our transport conditions.

### **ORF G confers MTX resistance and is overexpressed in folate transport mutants**

Mutations in the common high affinity folate/MTX transporter are a frequent mechanism of resistance to MTX in *Leishmania* (Ellenberger and Beverley, 1987b; Kaur *et al.*, 1988; Papadopoulou *et al.*, 1993). Similarly, mutation in the reduced folate carrier is a common MTX resistance mechanism in cancer cells (reviewed in Gorlick *et al.*, 1996; Kamen, 1997). We show here that in *L.tarentolae* MTX-resistant mutants in which there is no measurable high affinity folate/MTX uptake, the *orfG* gene is rearranged leading to an increased biopterin accumulation in the cell (Figure 5A and C). Remarkably, the overexpressed ORF G can also transport folic acid, albeit less efficiently, but it does not transport MTX (Figure 2). The folate requirements of these mutants may therefore be met by ORF G, provided that cells are grown in a folate-rich environment such as in SDM-79 medium. Recently, mutations in the mammalian reduced folate carrier were associated with increased affinity for folic acid and decreased affinity for MTX (Zhao *et al.*, 1998), showing that pteridine transporters can also discriminate between the two closely related substrates folic acid and MTX. An increase in the intracellular concentration of biopterin mediated by overexpression of *orfG* is likely to translate into an increase in reduced folate synthesis (Figure 6) and should also contribute to the folate requirements of cells which have lost their high affinity folate transporter.

The ORF G was isolated by selecting for MTX resistance and indeed, *L.tarentolae orfG* transfectants and the *orfG*-null mutants are resistant or hypersensitive to MTX, respectively (Figure 4C). Resistance mediated by overexpression of ORF G is likely to be due to both increased biopterin uptake and to increased selective folate uptake. The SDM-79 medium used to grow our cells contains a high concentration of folates (13  $\mu$ M), which makes it possible that the selective increase in ORF G-mediated folate uptake contributes to resistance in the MTX-resistant cells that have lost their high affinity folate/MTX transporter. Indeed, at high folate concentration (6  $\mu$ M) we can observe folate transport in MTX 1000.6 whereas no transport can be measured when lower concentrations of folates are used (Figure 2D). Unpublished ongoing experiments clearly show, however, that the increased uptake of biopterin and its putative conversion into folic acid also contributes significantly to the MTX resistance phenotype. For example, *orfG* overexpression confers MTX resistance in media in which the folate concentration (<150 nM) is too low (Figure 2) to be transported significantly by ORF G (unpublished). Resistance in these cells is likely to be due to the increase in biopterin uptake and its subsequent conversion into folates. Overexpression of ORF G in a *ptr1*-null mutant which cannot synthesize reduced pterins does not lead to MTX resistance even when folic acid or biopterin are supplemented to the cells (unpublished). However, when dihydrobiopterin is

supplemented to the same cells, we observed a >100-fold increase in MTX resistance (unpublished) which is probably due to an increase in the *de novo* biosynthesis of folates (Figure 6).

The increased activity of ORF G was not associated with gene amplification, but an excellent correlation was observed between the increased biopterin transport phenotype and a DNA rearrangement within the *orfG* locus (Figure 5A). In the mutant TarII MTX1000.6, we observed that the gene rearrangement was correlated with an increase in the steady-state level of the transcript (Figure 5B). The size of the transcript is different than in wild-type cells and may be a consequence of the rearrangement. Translocation of the *orfG* gene in the ribosomal locus has been demonstrated before, resulting in higher levels of *orfG* RNA (Lodes *et al.*, 1995). Trypanosomatidae are capable of expressing their genes by an RNA polymerase I (Rudenko *et al.*, 1991; Zomerdijk *et al.*, 1991). The rearrangement observed in this study is different, however, from the one observed in the ribosomal locus. The increased expression of a gene correlating with gene rearrangement rather than gene amplification is likely to have occurred in at least one other drug-resistant mutant (Gammaro *et al.*, 1994).

In conclusion, functional cloning has led to the isolation of a novel MTX resistance gene which was found to be involved in the resistance phenotype of drug resistant mutants. Interestingly, we showed that the loss of a high affinity transporter that is required to resist a drug analog (MTX) is compensated by the overexpression of another transporter. Transport-related mechanisms are frequent in drug-resistant organisms (Borst and Ouellette, 1995) and it is therefore possible that reduced uptake in these organisms is compensated by modulating the activity of other transporters.

## **Materials and methods**

### ***Leishmania* growth**

The *L.tarentolae* cell line TarII WT has been described previously (White *et al.*, 1988). *Leishmania* cells were grown in SDM-79 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 5 mg/ml of hemin (Brun and Schonenberger, 1979). MTX-resistant mutants of *L.tarentolae* and measurements of resistance levels have been described previously (Papadopoulou *et al.*, 1993, 1994a). *Leishmania tarentolae* promastigotes were transfected by electroporation as reported previously (Papadopoulou *et al.*, 1992).

### **DNA and RNA manipulations**

Chromosomes in agarose blocks were resolved by transalternative field electrophoresis (TAFE) (Beckman) as described previously (Grondin *et al.*, 1996). Total RNA was isolated using TRIzol (Gibco-BRL). Southern and Northern blotting, hybridization and washing conditions were performed following standard protocols (Sambrook *et al.*, 1989). Probes containing coding sequences of *orfG*,  $\alpha$ -tubulin and 18S rRNA were obtained by PCR.

### **Functional cloning**

A genomic cosmid library of partially *Sau3AI*-digested total DNA of *L.mexicana* in vector cL-Hyg (Ryan *et al.*, 1993) was constructed following a detailed protocol (Descoteaux *et al.*, 1994). After transfection of 10  $\mu$ g of the cosmid library DNA into *L.tarentolae* by electroporation (Bio-Rad Gene Pulser, voltage 0.45 kV and capacitance at 500  $\mu$ F), cells were propagated in SDM-79 medium overnight, grown for another 24 h in medium in which hygromycin B (Calbiochem) was added (200  $\mu$ g/ml), and then plated on SDM-79 agar plates containing 200  $\mu$ g/ml hygromycin B. After 10 days, the transfectants were pooled and multiplied in liquid SDM-79 medium containing 1 mg/ml of

hygromycin B. After 24 h the cells were collected and replated on 600 µM MTX-containing SDM plates. Several colonies grew on the MTX-containing plates, five of which were analyzed and found to contain an identical cosmid.

#### Generation of orfG expression vectors

The *Leishmania* expression vector pSL $\alpha$ neo $\alpha$  was constructed by insertion of a 2.5 kb *Bam*HI–*Bam*HI *neo* (neomycin phosphotransferase) expression cassette flanked by the  $\alpha$ -tubulin intergenic regions (Papadopoulos et al., 1992) into the single *Bam*HI site of the vector pSL1180 (Pharmacia Biotech). pSL $\alpha$ neo $\alpha$  was used to overexpress *orfG* from a 2.3 kb *Bgl*II–*Nhe*I fragment of *L.mexicana*, a 2.3 kb *Bgl*II–*Nhe*I fragment of *L.donovani* and a 2.7 kb *Pst*I–*Nhe*I fragment of *L.tarentolae*. To generate an ORF G–GFP fusion, pSL $\alpha$ neo $\alpha$  containing the 2.7 kb *Pst*I–*Nhe*I *orfG* fragment of *L.tarentolae* was linearized with *Nhe*I, digested with Mung Bean nuclease to remove 5' extensions of DNA and ligated to a 0.7 kb blunt end *Hind*III–*Xba*I fragment of pHGFP-S65T (Clontech Laboratories) containing the GFP gene. Correct in-frame fusion between *orfG* and GFP was confirmed by sequencing.

#### Construction of an orfG-null mutant

The 2.7 kb *Pst*I–*Nhe*I *L.tarentolae* fragment containing *orfG* was subcloned into pSP72 (Promega) and a hygromycin B phosphotransferase expression cassette (*hyg*) derived from pSPY-*hyg* (Papadopoulos et al., 1994b) was introduced into the unique *Csp*45I site within *orfG*. This *hyg*-containing 3.3 kb *Bgl*II–*Nhe*I fragment was used to disrupt one chromosomal *orfG* allele by homologous recombination. An *orfG*-null mutant was obtained by selection for loss of heterozygosity (Gueiros-Filho and Beverley, 1996) by increasing the hygromycin B selection pressure to 600 µg/ml and cloning of the cell pool. Double knock-out mutants were identified by Southern blot analysis.

#### DNA sequence analysis

DNA sequencing was done on an Applied Biosystems 373 DNA automated sequencer. Analysis of the sequence was performed using the GCG software package (Genetics Computer Group, 1994) and DNA Strider™ 1.0. The nucleotide sequence reported here will appear in the DDBJ/EMBL/GenBank sequence database under the accession No. AF078929.

#### Pteridine transport experiments

Transport experiments were performed as described (Papadopoulos et al., 1993). Tritium-labeled pteridines {[<sup>3</sup>H]folate (14.6 Ci/mmol), [<sup>3</sup>H]MTX (23.6 Ci/mmol), [<sup>3</sup>H]biopterin (5.8 Ci/mmol)} were purchased from Movarek Biochemicals. Transport studies were carried out with varying concentrations of radioactive pteridines (50 nM to 50 µM of biopterin, 50 nM to 20 µM of folate) to determine apparent kinetic parameters ( $K_m$  and  $V_{max}$ ) for folate and biopterin. The quantity of radioactivity incorporated was normalized with *Leishmania* cell numbers. We performed transport experiments with varying concentrations of the labeled pteridines. To measure biopterin transport in wild-type cells and in *orfG* transfectants, the accumulation of biopterin in an *orfG*-null mutant was subtracted, while for values of folate transport in wild-type cells and in *orfG* transfectants, uptake of cells incubated on ice have been subtracted. Data points used for the determination of kinetic parameters are the means of three experiments. Attempts to determine a  $K_m$  value for folate uptake mediated by ORF G failed due to insufficient sensitivity of the transport experiments. Significant folate uptake by ORF G is only detectable at high folate concentrations (>5 µM), which made it necessary to dilute the radiolabeled folic acid solution with large amounts of unlabeled folate. This lowered the absolute counts of radioactivity and made the calculations very susceptible to small errors in pipetting, which resulted in very large standard deviations after compensating the data for dilution factors, thereby rendering it impossible to measure accurately a  $K_m$  value for folate for the *L.tarentolae* ORF G.

To measure the effect of metabolic poisons on biopterin transport in *L.tarentolae*, wild-type cells within logarithmic growth phase were washed twice and incubated for 30 min at room temperature in 20 mM sodium azide or 5 mM 2,4-dinitrophenol, respectively. After treatment, the cells were used directly in transport experiments. Cells that were washed after drug exposure showed no significant decrease in transport, indicating that the majority of cells were not irreversibly damaged. This was also confirmed by the fact that treated cells did not show any retardation in growth when cultured in SDM-79 medium.

#### Fluorescence microscopy

*Leishmania tarentolae* cells expressing the ORF G–GFP fusion protein were grown in SDM-79 to late log phase, washed in phosphate-buffered

saline (PBS), fixed in 4% paraformaldehyde and resuspended in PBS at a density of  $\sim 10^7$  cells/ml. Specimens were viewed on a Bio-Rad MRC-1024 confocal imaging system equipped with a Krypton-argon laser beam and mounted on a Nikon Diaphot-TMD. A 60 $\times$  objective lens with a 1.4 numerical aperture was used. Confocal settings were as follows: 1 mW laser power, zoom 5, 1 s per scan Kalman filter and eight frames per image. The photomultiplier gain was set at maximum and the confocal aperture was adjusted for maximum resolution.

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