Description of a novel eukaryotic deoxyuridine 5′-triphosphate nucleotidohydrolase in *Leishmania major*

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A *Leishmania major* full-length cDNA encoding a functional dUTP nucleotidohydrolase (dUTPase; EC 3.6.1.23) was isolated from a cDNA expression library by genetic complementation of dUTPase deficiency in *Escherichia coli*. The cDNA contained an open reading frame that encoded a protein of 269 amino acid residues with a calculated molecular mass of 30.3 kDa. Although eukaryotic dUTPases exhibit extensive similarity and there are five amino acid motifs that are common to all currently known dUTPase enzymes, the sequence of the protozoan gene differs significantly from its eukaryotic counterparts. None of the characteristic motifs were readily identifiable and the sequence encoded a larger polypeptide. However, the products of the reaction were dUMP and PP_i, competition experiments with other deoxyribonucleoside triphosphates showed that the reaction is specific for dUTP, and the protozoan gene complemented dUTPase deficiency in *Escherichia coli*. The gene is of single copy; Northern blots indicated a transcript of the same size as the cDNA isolated in the screening procedure. The enzyme can be efficiently overexpressed in a highly active form by using the expression vector pET-11c. The availability of recombinant enzyme in large quantities will now permit detailed mechanistic and structural studies, which might contribute to a rational design of specifically targeted inhibitors against dUTPase from *L. major*.

INTRODUCTION

Apart from cytosine deamination, uracil can be incorporated into DNA through the utilization of dUTP by DNA polymerase. dUTP is an intermediate compound in the biosynthetic pathway of dTTP and is formed in all eukaryotic dividing cells from UDP via ribonucleotide reductase and nucleotide diphosphate kinase. If the intracellular levels of this compound increase, in the presence of unbalanced ratios of dUTP to dTTP, uracil incorporation into DNA can be facilitated [1] and induction of the uracil glycosylase-mediated repair can occur. During repair, uracil is likely to be reinserted, causing a futile cycle of excision repair and reinsertion, leading to strand breakage and ultimately cell death [2]. Under normal conditions, the levels of dUTP are kept low by the action of dUTP nucleotidohydrolase (dUTPase; EC 3.6.1.23), a ubiquitous enzyme that catalyses the hydrolysis of dUTP to PP_i and dUMP, a substrate for thymidylate synthase (TS). The enzyme is essential for viability in *Escherichia coli* [3] and *Saccharomyces cerevisiae* [4], which suggests that activity might be crucial for DNA replication and cell division. Studies in human T-cells have demonstrated that dUTPase was induced with proliferation in mature T-cells and constitutively expressed in immature thymocytes, and that accumulation and phosphorylation of the enzyme in mature cells occurred in a cell cycle-dependent manner [5]. Two distinct forms of the enzyme exist in human cells: a lower-mass, more abundant, form that localizes in the nucleus and a higher-mass form associated with the mitochondria [6]. The nuclear form might be a target for cyclin-dependent kinase phosphorylation *in vivo* [7]. The association of dUTPase activity with proliferation has also been described in higher plants, where dUTPase levels increase in proliferating root meristems in a cell cycle-dependent manner [8]. Evidence from studies in *Drosophila* indicated that the regulation inhibition of dUTPase [9], combined with the differential expression of uracil glycosylase, might lead to programmed cell death during larval development [10].

Metabolism of dUTP has been clearly related to the phenomenon of thymine-less death. Although the exact mechanism of this process has not been defined, there is evidence indicating that one component of the toxic effects of inhibition of dTMP synthesis is the active incorporation of uracil into DNA [11,12]. As a consequence of the accumulation of dUMP, dUTP accumulates, exceeds the hydrolytic capacity of dUTPase and is used by DNA polymerase for incorporation into DNA. Canman et al. [13] have demonstrated that resistance to fluorodeoxyuridine-induced damage is correlated with elevated dUTPase levels and a lack of accumulation of dUTP. Likewise, expression of *E. coli* dUTPase in an established tumour cell line confers resistance to fluoropyrimidines [14]. On the basis of these findings and the observation that substrate-analogous inhibitors of dUTPase selectively inhibit the proliferation of human cancer cells *in vitro* [15], it has been suggested that the enzyme might represent a target for drug development.

The need for investigation into this particular area of DNA metabolism and the potential interest of dUTPase has prompted us to investigate the fate of dUTP in *Leishmania* cell extracts. We also describe the isolation of a cDNA encoding for a novel functionally active dUTPase that hydrolyses dUTP to $dUMP + PP_i$ but that apparently lacks the characteristic domains of other eukaryotic dUTPases so far described.

Abbreviations used: CHEF, contour-clamped homogeneous electric field; DHFR, dihydrofolate reductase; dCTPase, dCTP nucleotidohydrolase; dUTPase, dUTP nucleotidohydrolase; ORF, open reading frame; PEI, poly(ethyleneimine); TS, thymidylate synthase.

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The nucleotide sequence reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number L76742.

EXPERIMENTAL

Reagents

Deoxy[5-^{3}H]uridine 5′-triphosphate (15 Ci/nmol) ammonium salt was purchased from Amersham. Poly(ethyleneimine) (PEI)–cellulose plates with a fluorescent marker were obtained from Whatman. 2′-Deoxyuridine 5′-triphosphate sodium salt, 2′-deoxyuridine 5′-diphosphate sodium salt, 2′-deoxyuridine 5′-monophosphate sodium salt and the other nucleosides triphosphate, aprotinin, trypsin inhibitor, sodium phosphate, BSA, dithiothreitol and the kit for determination of P_i were from Sigma. PMSF and leupeptin were purchased from Boehringer Mannheim. The *E. coli* strain BW286 [*dut-1,* Δ(*xth-pncA*)90] used to screen the *Leishmania major* cDNA library has been described [16] and was supplied by Dr. Bernard Weiss (John Hopkins University, Baltimore, MD, U.S.A.). The ZAP Express® system for the construction of the library and the pET expression system were purchased from Stratagene. Oligonucleotides ATG-dut (5′-GGAATTCCATATGAAGCGCGCTCGCAGC-GC-3′) and TAA-dut (5′-CGCGGATCCTTATGCCTTGAT-CGCCAGCCG-3′) were synthesized at the Analytical Services of the Instituto de Parasitologia y Biomedicina 'López-Neyra', Granada.

Preparation of cell extracts

L. major promastigotes were collected, centrifuged (1430 ***g*** for 10 min at 4 °C) and washed with PBS buffer. The pellets were frozen and stored at −80 °C. Frozen *L. major* pellets were thawed and resuspended in a buffer containing 50 mM Tris/HCl, 5 mM dithiothreitol and 4 mM $MgCl_2$, with 50 μg/ml aprotinin, 20 μg/ml leupeptin, 50 μg/ml trypsin inhibitor and 50 μM PMSF as protease inhibitors. Cells were sonicated and centrifuged for 30 min at 12000 ***g***; the supernatant was used for measurements of dUTP hydrolysis activity.

Assay of dUTP hydrolysis

The assay measures the production of [^{3}H]dUMP and [^{3}H]dUDP from [^{3}H]dUTP [12]. The reaction mixture contained, in a total volume of 20 μl, 150 mM Tris/HCl, pH 7.5, 25 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 3% (w/v) BSA, 0.1 mM dUTP (100–500 mCi/mmol) and crude extract (3–6 μg). Unlabelled nucleotides for competition experiments were added to the assay mixture as indicated in each case. All the assays were incubated for 10 min at 37 °C after preincubation for 5 min at the same temperature. The reaction was stopped by the addition of EDTA to a final concentration of 50 mM. The reaction mixture (3.5 μl) was loaded on a TLC PEI–cellulose plate together with 2 μl of a solution containing dUDP and dUMP (each at 6 mM), to provide optical markers under UV irradiation. The PEI–cellulose plates were prewashed with 10% (w/v) NaCl solution and washed twice with distilled water. Plates were air-dried overnight and stored in the dark at 4 °C until needed. The washed plates were divided into channels and the chromatography sheets were developed in 1 M LiCl, which was allowed to migrate to 10 cm from the origin, positioned at 2 cm from the bottom of the plate. The sheets were air-dried at room temperature and examined under UV irradiation. The spots containing deoxyuridine nucleotides were cut out and eluted from PEI–cellulose with 1 ml of 0.2 M sodium phosphate. Aquasol-2® (10 ml) and 1 ml of distilled water were added; radioactivity was determined in a liquid-scintillation counter.

L. major cDNA library and phagemid rescue

The cells used for construction of the cDNA library were promastigotes derived from the 252 strain of *L. major*. Cells were grown in M199 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco). Exponential-phase (17×10^6 parasites/ml) were used. Isolation of poly(A)$^+$ RNA was performed with the QuickPrep Micro mRNA Purification Kit (Pharmacia). Phagemids were rescued from the library by co-infection of *E. coli* XLIB with 2×10^8 plaque-forming units of λ phage and 10^{10} plaque-forming units of ExAssist helper phage in 25 ml of Luria–Bertani medium. The supernatant obtained after incubation and clarification of culture by centrifugation had a titre of 2.5×10^3 kanamycin-resistant colony-forming units/μl. The isolation of plasmid DNA was accomplished with the Wizard® Maxipreps DNA Purification System (Promega). The mutant strain BW286 was used to screen the *L. major* cDNA library. Sequencing was performed with PCR with fluorescent dye-dideoxy terminators. General methods for DNA manipulations were as described [17].

Northern and Southern blot analysis

Poly(A)$^+$ RNA was subjected to electrophoresis on 1% (w/v) agarose gels containing 1 × Mops buffer [20 mM Mops/0.08 M sodium acetate (pH 7.0)/1 mM EDTA] and 6.29% (v/v) formaldehyde after the samples had been boiled for 10 min in 50% (v/v) formamide/1 × MOPS buffer/5.9% (v/v) formaldehyde. A 0.24–9.5 kb RNA ladder (Gibco) was used as a standard. The gel was transferred to a Hybond-N filter and hybridized with the dUTPase gene. The probe used in hybridization studies was prepared by PCR with oligonucleotides complementary to nucleotide positions +1 to +20 (5′ primer) and +787 to +807 (3′ primer) of the dUTPase cDNA sequence. For Southern blot analysis, total *L. major* genomic DNA was isolated by phenol extraction, digested with different endonucleases, transferred to a Hybond-N filter and hybridized with the dUTPase probe.

Contour-clamped homogeneous electric field (CHEF) electrophoresis

Low-melting-point agarose blocks of *L. major* were prepared as described [18]. Chromosomes were separated on a 1% agarose gel in 0.5 M Tris/borate/EDTA/0.5 mg/ml ethidium bromide with a CHEF electrophoresis system (Pharmacia). The parameters used were 40 h at 160 V with a pulse time of 45 s. The resulting gel was transferred to a Hybond-N (Amersham) nylon filter and subjected to Southern blot analysis. Agarose electrophoresis and hybridization were performed by standard procedures.

Expression system construction

For expression in *E. coli* the entire coding sequence was amplified by the PCR technique. The reaction mixture (50 μl) contained 25 pmol of each of the two oligonucleotide primers ATG-dut and TAA-dut, 100 ng of pBKLMDUT DNA, dNTPs (each at 400 μM), 60 mM KCl, 25 mM Tris/HCl, pH 8, 10 mM $MgCl_2$ and 0.1% BSA. Amplification was initiated with 1.5 units of *Taq* polymerase. PCR parameters were 35 thermal cycles consisting of a 60 s denaturation at 94 °C followed by a 90 s annealing period of 65 °C and a 2 min extension period at 72 °C. Oligonucleotide primers for amplification of the dUTPase coding sequence (ATG-dut and TAA-dut) were designed so that *Nde*I and *Bam*HI restriction sites were introduced at the 5′ and 3′ ends for convenient cloning in the expression vector pET-11c to give

pETLMDUT, which was originally cloned and propagated in XL1B. Double-stranded DNA sequencing was performed to confirm the correct sequence after amplification. Subsequently, pETLMDUT was used to transform the *E. coli* expression host BL21(DE3). Bacterial clones were grown in Luria–Bertani medium containing 50 mg/ml ampicillin. When induction was performed, bacterial cells transformed with pETLMDUT were first grown to an absorbance of 0.6 at 37 °C and then 1 mM IPTG was added; cultures were then grown for 2.5 h at 37 °C, cells were collected by centrifugation and, when not used immediately, frozen at −80 °C.

RESULTS

Analysis of dUTP hydrolysis in *Leishmania* cells

In order to investigate the existence of dUTPase activity in *L. major*, cell extracts obtained by sonication were incubated with [^{3}H]dUTP and the resulting products were analysed by TLC. Both dUMP and dUDP were formed from dUTP in the presence of cell extracts, yet no radioactivity was detected in the front of the plates where uridine or uracil would normally migrate and therefore under the present conditions did not appear in measurable amounts. As shown in Table 1, several deoxyribonucleoside triphosphates were tested for their dilution effect on dUTP hydrolysis. dATP, dGTP, dTTP, dCTP and dUTP were added to the incubation mixture at 0.5 mM; the concentration of [^{3}H]dUTP was maintained at 100 μM. Only dUTP decreased the amount of radioactivity found in the dUMP fraction, suggesting that the formation of the deoxynucleoside monophosphate occurs in a reaction that is specific for dUTP and is not due to a non-specific phosphatase or nucleotidase activity present in the cell-free system.

Isolation of *Leishmania* dUTPase

The *dut* gene was isolated from a *L. major* ZAP Express cDNA expression library by complementation of dUTPase deficiency in *E. coli*. The bacterial strain used for screening (BW286) contains both the *dut-1* (dUTPase) and Δ(*xth-pnc*A)90 (exonuclease III) alleles, which in combination are lethal on rich medium at 37 °C [3]. The library was converted by excision *in vivo* to the pBK-CMV phagemid form and supercoiled plasmid DNA was used for screening. A plasmid equivalent of the cDNA library was generated by the excision of inserts and adjoining plasmid sequences from ZAP Express recombinants and packaging of their single-strand equivalents into phagemids. XLOLR cells were infected with 12×10^6 plaque-forming units, and plasmid DNA from the total pool of resulting colonies was used to transform BW286 cells. A total of 25×10^4 colonies were tested for complementation. Eleven plasmids were isolated that complemented the temperature-sensitive phenotype of BW286. One of them (pBKLMDUT) exhibited a 2.0 kb insert and complemented conditional lethality at a high frequency when a second transformation of temperature-sensitive cells was performed. Measurements of dUTP hydrolysis in crude extracts of transformed cells and analysis of the products revealed that dUMP was the only detectable reaction product and that dUTPase activity was largely increased compared with non-transformed cells grown at 28 °C (Table 2). Furthermore, competition experiments with different nucleotides (Table 1) demonstrated that the hydrolytic activity was specific for dUTP.

The insert was fully sequenced and analysed for the existence of open reading frames (ORFs) showing homology with dUTPase sequences from other sources (Figure 1). Both the spliced leader and the poly(A) tail were present at the 5′ and 3′ ends respectively. An ORF of 807 bp was located that encoded a protein of 269 residues and a calculated molecular mass of 30.3 kDa that was initiated with the first ATG identified in the cDNA sequence. The ORF presented a codon usage that correlated closely with that determined for other *Leishmania* genes and was the longest identified. Other possible ORFs were either in the same reading frame or were considerably shorter (291 bp) and presented an unusual codon usage. The deduced amino acid sequence was analysed for similarity to sequences of known dUTPases. The primary sequence of the enzyme is highly conserved among eukaryotes (56 % identity between the human and *S. cerevisiae* enzymes) and there are five amino acid consensus sequence motifs that are common to most dUTPases characterized so far [19]. Surprisingly the *Leishmania* enzyme seemed to be substantially different from other eukaryotic dUTPases. Similarity to the human [20] and *yeast* [4] sequences was not significant and the amino acid consensus sequences were not easily identified. The protein encoded by the ORF is larger than the human (141 residues), *E. coli* (150 residues) [21] and *S. cerevisiae* (147 residues) enzymes, although it approximated the size of Epstein–Barr virus [22] dUTPase, which contains 278 amino acid residues.

Northern blot analysis and genomic organization of the dUTPase gene

A Northern blot of poly(A)$^+$ RNA extracted from both epimastigote forms of the parasite was performed by using the dUTPase cDNA as probe. The analysis indicated a transcript size of 2.0 kb (Figure 2). Prolonged exposure of the autoradiogram did not reveal any additional bands and therefore the size of the mRNA determined by cDNA analysis corresponds to the form observable in a Northern blot.

Table 1 Effect of dNTPs on dUTPase activity

The addition of unlabelled 0.5 mM dNTPs was evaluated in crude extracts of *L. major* and in BW286 cells transformed with the expression plasmid pBKLMDUT, which contains the *L. major* dUTPase gene. Only dUTP was found to produce a dilution effect on dUTP hydrolysis. Results are means ± S.E.M. for dUTPase activities determined in three independent incubations.

Addition	dUTPase activity (nmol/min per mg of protein)	
	Leishmania crude extracts	*E.coli* BW286/pBKLMDUT
None	2.84 ± 0.12	95.92 ± 10.72
dUTP	0.12 ± 0.08	9.48 ± 0.39
dATP	2.84 ± 0.12	108.94 ± 0.93
dTTP	3.21 ± 0.29	113.91 ± 11.70
dGTP	3.68 ± 0.22	124.52 ± 3.01
dCTP	3.64 ± 0.37	123.78 ± 1.12

Table 2 dUTPase activity in cell extracts

Enzyme activity was determined in *Leishmania* crude extracts and in *E. coli* cells transformed with the different expression plasmids. Results are means ± S.E.M. for dUTPase activities determined in three independent incubations.

Cell/plasmid	dUTPase activity (nmol dUMP/min per mg of protein)
L. major/ —	3.21 ± 0.31
E. coli BW286/pBKLMDUT	177.86 ± 31.48
E. coli BL21(DE3)/pETLMDUT	2360.87 ± 90.02

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-91  ATCAGTTTCTGTACTTTATTGTTCAAATCCCCTACTTGCTCGCTCACAATAGAAGCATA

-32  AAACTACTGCACTCAAAAAGGGAAAAAGACG ATG AAG CGC GCT CGC AGC GCC
                                     1▶Met Lys Arg Ala Arg Ser Ala

 21  AAT ATT CCA GGT GCG ATT CTG CAC TCC TTG GCA GAG CTG CAG GAT
  8▶Asn Ile Pro Gly Ala Ile Leu His Ser Leu Ala Glu Leu Gln Asp

 66  GGC CTG AAC GCG ATG ATC GAC CCT TCC TGG CGC GCT GTT CGC TCG
 23▶Gly Leu Asn Ala Met Ile Asp Pro Ser Trp Arg Ala Val Arg Ser

111  CTG GAC AAT TGG GCT CTA GCC ATC ACG ATG GAG TCG ACG GAG CTG
 38▶Leu Asp Asn Trp Ala Leu Ala Ile Thr Met Glu Ser Thr Glu Leu

156  CTG GAC TCC TAT CCG TGG AAG TGG TGG AAG AAC CTC AAC GCT ACG
 53▶Leu Asp Ser Tyr Pro Trp Lys Trp Trp Lys Asn Leu Asn Ala Thr

201  CCT GAT TTG GCC AAC GTT AGG ATT GAA CTG GTC GAC ATC TTT CAC
 68▶Pro Asp Leu Ala Asn Val Arg Ile Glu Leu Val Asp Ile Phe His

246  TTT TCC CTT TCG GGC GCT ATG CAA ATG CGG TCT ACC CCG GAC GAT
 83▶Phe Ser Leu Ser Gly Ala Met Gln Met Arg Ser Thr Pro Asp Asp

291  GAG ATT CCC GCC GCT TCG CTC AAG CCT CTG AAG GAG GTC ATG ACA
 98▶Glu Ile Pro Ala Ala Ser Leu Lys Pro Leu Lys Glu Val Met Thr

336  ACT TTT CTG CCG GCT AAG GAG TGC ACG AGC GAC CCT TAC GGC TTT
113▶Thr Phe Leu Pro Ala Lys Glu Cys Thr Ser Asp Pro Tyr Gly Phe

381  GTG TTC TTT CCC CTC ACC GAT ACA CAG AAC GCC ATT GCG AGC TTT
128▶Val Phe Phe Pro Leu Thr Asp Thr Gln Asn Ala Ile Ala Ser Phe

426  CGT AAC ATC ATT CAG CTC GCA AAC GCC TAT CGC TTC GAT GTC ATC
143▶Arg Asn Ile Ile Gln Leu Ala Asn Ala Tyr Arg Phe Asp Val Ile

471  ATA GAG TGC ATC ATA TAC GCC GCT GAG GAC CTG GGG TTT AAC TTG
158▶Ile Glu Cys Ile Ile Tyr Ala Ala Glu Asp Leu Gly Phe Asn Leu

516  GTG GCG TAC TAT ATT GCA AAA CAC ACT CTG AAC TGC ATT CGC CAG
173▶Val Ala Tyr Tyr Ile Ala Lys His Thr Leu Asn Cys Ile Arg Gln

561  CTG AGT GGC TAC AAA GAC GGC TCT TAC GTG AAG GTG AAC AAT GGT
188▶Leu Ser Gly Tyr Lys Asp Gly Ser Tyr Val Lys Val Asn Asn Gly

606  GTT GAG GAC AAT TCA CTT CTG CAC AAC TGC ATC AAA GAC GTA TCC
203▶Val Glu Asp Asn Ser Leu Leu His Asn Cys Ile Lys Asp Val Ser

651  CTC GAT GAA GTT CTC GAC GCT GAC AAG TAT GTC CAG GCG TGG AAC
218▶Leu Asp Glu Val Leu Asp Ala Asp Lys Tyr Val Gln Ala Trp Asn

696  AGC ATC ATG GCT AAC GTT TAC GAA GCT TTC CAG ATT AAG GAG TCT
233▶Ser Ile Met Ala Asn Val Tyr Glu Ala Phe Gln Ile Lys Glu Ser

741  GAC CGA AAG GAT GCG GAG CGC TGG TTT GCC CTC GCA AAG GAA AAT
248▶Asp Arg Lys Asp Ala Glu Arg Trp Phe Ala Leu Ala Lys Glu Asn

786  CGG CTG GCG ATC AAG GCA TAA AGCTCTTCTTCCCAAATCCTCTCATGCGAAA
263▶Arg Leu Ala Ile Lys Ala •••

838  GGATCGACTTTTGTTTTCTGCTTAACTACCAAGCTTCAGACAGTCAAGTTGTAGACATG

897  CCTTTTCTTCCGACATCCATTTTACGCCTTTAGGCGAGAGCTCATCGAGGAGGTAAGCG

956  CTTCTTTCTCCCCTCTTTTTCGTCTCTTCGATGTCTTGCTTCCGCCATAAACGTATCC

1015 ATCGAGGATCTTTCTCCTGAAAGGGTCAGAGGATCTCATCGATATGAAGTGAAAGTGCT

1074 CACCGCTTTTCGGCTCTGTTTCGCTCATCCATGTCCATTTCCTTTGCTATATGTATATA

1133 TATATATATATATACATAAAGACATAGAATTTTATAAAGGAAGGAGGGAAACGAAAA

1192 GAAAGATAAGCGAGGCGGTCGAACGGCCGTGCCCTTCTCAAGGACAATAGAAACAAAAA

1251 AACAAACATGAAGCTCAACACTGTGAAATGAAGAGTTCATCTGATTTTATTTTGCGTTT

1310 TTGCACACGGTCTTCACCCACGTGTTTGGCACGTTGGAAGGGTGTTTTCAAAACGTGTG

1369 ATTGGGTTTCGGGGATTCTGCCTCAACGGCGTGATTCCCCTATCTGCGGCCACAACATC

1428 GTGAGGGTAAATCTAACCGGATGTTTTCCCCCTTTGACTACACTATTTCTCGCCCAAT

1487 ACCTCTGCACCAGCGGATTGCAGCTCAAGCATCACACCGAACGTTTCAAACGCAGCAGC

1546 CCGTTGCGAGCTAACTGGATATCTAACAGGGAATACCTTGCAGCTGGCTTGATTCTCAC

1605 TTTTATCCTTACAGGAGCAGATTCAGTGGAATACCTTGAAGGCTTGTTAGTTCTCCTTG

1664 CTCGACAAACTTACAGGGTGTGGGCGATGTTTCAAACAACATCTGCGTTCGATAAAAGC

1723 AATAGGTTTGCTTAGACGAATATGCTGAGTCTGCTCTTCCCTTTTTGTTCGAGAGTCTG

1782 CGTGGGCCCCTCATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1 cDNA sequence of the *L. major* dUTPase gene

The location of the splice leader, a 39-base 5′ sequence that is present in all coding RNAs in trypanosomatids, is underlined. The predicted amino acid sequence of the *Leishmania* enzyme derived from the ORF is shown under the nucleotide sequence.

Genomic DNA from *L. major* 252 cells was digested with different endonucleases, blotted and hybridized with the dUTPase gene probe prepared as described in the Experimental section (Figure 3). Lane a corresponds to a digestion with *Sac*I, which cuts within the cDNA sequence, and lane b is a digestion with *Nae*I, a unique restriction site present in the coding region of the dUTPase gene. Lanes c and d in Figure 3 correspond to

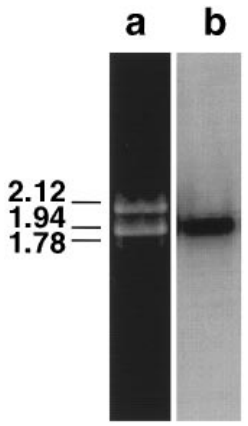

Figure 2 Northern blot analysis of dUTPase transcripts

Northern blot analysis of the *L. major* dUTPase transcript with a ^{32}P-labelled specific probe. Approx. 7 μg of poly(A)$^{+}$ RNA were subjected to electrophoresis on the gel before transfer to nylon. Lane a, poly(A)$^{+}$ RNA from *L. major*; lane b, autoradiograph obtained after transfer of the gel shown in lane a and hybridization with the dUTPase probe.

digestions with *Bgl*II and *Xho*I (sites not contained within the cDNA sequence). The sizes of the resulting bands agree with the restriction map obtained from the cDNA clone; the number of bands observed in the digests suggests that the gene is of single copy.

Expression of *L. major* dUTPase in *E. coli*

Complementation of the temperature-sensitive phenotype in the cDNA screening demonstrated that functional *Leishmania* dUTPase was synthesized. The specific activity in *E. coli* cells transformed with pBKLMDUT was approx. 55-fold the activity detected in *Leishmania* crude extracts (Table 1). In SDS polyacrylamide gels of *E. coli* cells transformed with pBKLMDUT, a band representing approx. 2 % of the total protein was evident. However, further work on *Leishmania* dUTPase depends on the ability to generate sufficient quantities of the enzyme to perform a detailed structural and kinetic characterization. To enhance the

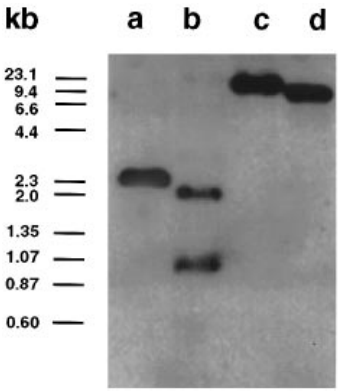

Figure 3 Southern blot analysis of genomic DNA from *L. major*

Total genomic DNA was digested with *Sac*I (lane a), *Nae*I (lane b), *Bgl*II (lane c) or *Xho*I (lane d). The DNA fragments were separated on a 1% (w/v) agarose gel, transferred to nylon and hybridized with the dUTPase probe.

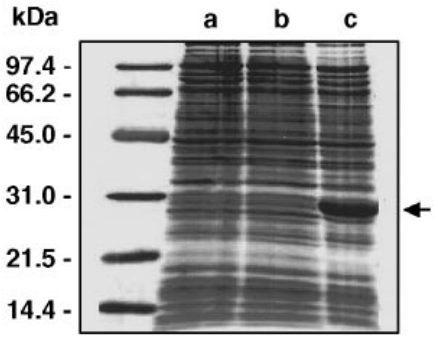

Figure 4 SDS/PAGE analysis of *L. major* dUTPase expression in *E. coli*

Lane a, 20 μg of crude extract from IPTG-induced *E. coli* BL21(DE3), pET-11c; lane b, 20 μg of crude extract from IPTG-non-induced *E. coli* BL21(DE3), pETLMDUT; lane c, 20 μg of crude extract from IPTG-induced *E. coli* BL21(DE3), pETLMDUT. The gel was stained with Coomassie Blue. Molecular mass standards are shown at the left.

level of expression, the entire coding region identified was amplified by PCR and placed in the expression vector pET-11c (Stratagene) to give pETLMDUT, which was used to transform BL21(DE3) cells. Lysates of cells induced for 2.5 h with IPTG displayed a 30 kDa band not present in cells transformed with the vector alone (Figure 4). Densitometric scanning of stained gels showed that the level of *Leishmania* dUTPase was approx. 15% of soluble protein in *E. coli* containing the expression construct. Enzyme activity determinations indicated that specific activity was approx. 13-fold that obtained with BW286 cells transformed with pBKLMDUT (Table 1). The products of the reaction were shown to be dUMP and PP_i. For dUMP, TS activity dependent on dUTPase [23] was demonstrated by incubating dUTP with methylene-tetrahydrofolate and purified *Lactobacillus casei* TS as described [24], initiating the reaction with extracts of *E. coli* BL21(DE3) cells transformed with pETLMDUT. In addition, treatment of the reaction products with pyrophosphatase and further determination of P_i also corroborated the appearance of PP_i (results not shown).

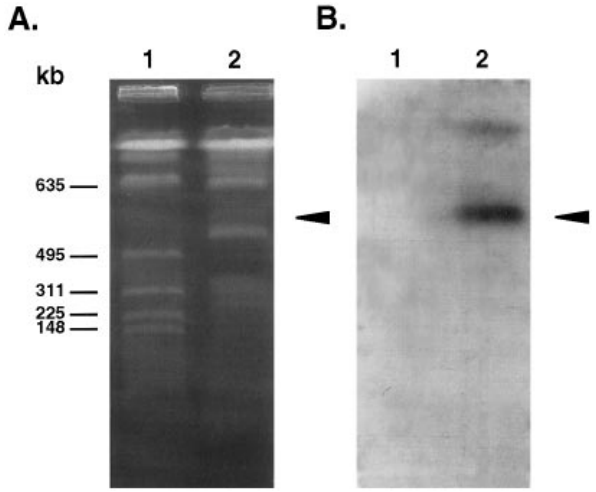

Figure 5 Chromosomal location of the *Leishmania* dUTPase gene

(**A**) Ethidium bromide-stained gel of a CHEF electrophoresis from *L. major*. Lane 1 corresponds to *S. cerevisiae* as molecular mass marker chromosomes; lane 2 corresponds to *L. major* chromosomes. (**B**) Autoradiograph of a nylon transfer of the gel shown in (**A**), probed with the coding sequence of the dUTPase gene. Arrows show the location of the *L. major* dUTPase gene.

Chromosomal localization of the *dut* gene

The chromosomes of *Leishmania* range in size between 200 and 2000 kb [25] and can be resolved by CHEF electrophoresis. The *Leishmania* dUTPase probe was used to hybridize a filter replicate of chromosomes of *L. major* under conditions that permitted an increased resolution of small chromosomes. As shown in Figure 5, dUTPase sequences were located on a single chromosome identified as chromosome 6. We confirmed that dihydrofolate reductase (DHFR)–TS sequences were found on the same chromosome, suggesting a possible genetic linkage between both genes, which are involved in related functions in pyrimidine metabolism.

DISCUSSION

In protozoa much attention has been directed towards TS and DHFR, which catalyse sequential reactions in the synthesis of dTMP and are parts of a single polypeptide chain with the DHFR domain on the N-terminus and the TS domain on the C-terminus. The characterization of the bifunctional enzyme has been accomplished in several protozoan species [26–29] with the idea that a detailed knowledge of unique characteristics in these organisms would lead to selective inhibition and the development of a drug to treat parasitic diseases. DHFR inhibitors such as trimethoprim and pyrimethamine have been widely used as antimalarial agents and the inhibition of growth of *Leishmania* by methotrexate has been studied in detail; forms resistant to the drug have provided an insight to the molecular mechanisms involved in drug resistance [18,30,31]. Although the lethal effects of methotrexate and the essential role of DHFR–TS have been clearly demonstrated in *Leishmania* [32], the nature and role of dUTP metabolism have not been analysed. In mammalian cells, inhibitors of either enzyme activity have been shown to profoundly perturb uracil metabolism, promoting a pronounced increase in intracellular dUTP concentrations and the incorporation of uridylate into DNA. The activation of the base excision repair mechanism might ultimately inhibit daughter strand synthesis or induce both single-strand and double-strand breaks in DNA [33]. The first line of defence against the incorporation of dUMP into DNA by DNA polymerase is dUTPase, which maintains dUTP concentrations at 10^{-5} times that of dTTP [11], thereby making dUTP unavailable to the DNA polymerase.

Initial determinations performed in crude extracts and competition experiments with other pyrimidine and purine deoxyribonucleoside triphosphates supported the existence of dUTPase activity in *Leishmania* cells. The purpose of the competition studies was to determine whether hydrolysis of dUTP was a non-specific process due to a phosphatase activity present in the crude extract or a reaction catalysed by dUTPase. Formation of dUMP was apparently specific for dUTP, which was in good agreement with what has been established for dUTPases from other sources.

Although dUTPase activity was clearly present in cell extracts, attempts to isolate *L. major* dUTPase gene sequences by PCR or low-stringency hybridization with cloned sequences as probes were unsuccessful. Therefore an approach was made to isolate a *L. major* dUTPase cDNA sequence by direct expression in *E. coli*. A similar strategy was used in the isolation of the human [20] and yeast enzymes [4]. The cloning of the dUTPase by genetic complementation is, to our knowledge, the first example of cloning of a trypanosomatid gene by direct expression in a

77	E	L	V	D	I	F	H	F	S	L	S	*L. major* dUTPase
130	E	L	V	D	I	F	H	F	V	L	N	T4 dCTPase
59	W	K	W	W	K							*L. major* dUTPase
103	W	K	K	W	K							T4 dCTPase
72	L	A	N	V	R							*L. major* dUTPase
46	L	A	N	V	R							T4 dCTPase

Figure 6 Comparison of amino acid motifs from the *L. major* dUTPase and T4 dCTPase sequences

The T4 dCTPase sequence was extracted from Swissprot release 32.

heterologous system. Of a total of 11 positive clones that complemented the temperature-sensitive phenotype, only one exhibited dUTPase activity. Characterization of the remaining 10 clones revealed that all of them contained an ORF encoding a protein highly similar to type II apurinic/apyrimidinic endonucleases, of which *E. coli* exonuclease III is a representative (J. Pérez, V. Bernier, A. Camacho, D. González-Pacanowska and L. M. Ruiz-Perez, unpublished work). An examination of the 5′ flanking region revealed a purine-rich sequence (AGGGA) positioned at −10 to the translation initiation codon; this might have contributed to the efficient translation of the dUTPase gene in *E. coli*. Although the coding sequence isolated clearly presents dUTPase activity when expressed as a recombinant protein, the amino acid sequence deduced was markedly different from all eukaryotic dUTPases so far characterized. Database searching revealed the existence of partial sequence similarity to dCTP nucleotidohydrolase (dCTPase) of the bacteriophage T4 and characterized as gp56 [34]. This enzyme exhibits both dUTPase and dCTPase activities and is part of the T4 deoxyribonucleoside triphosphate synthetase complex whose role is the production of deoxyribonucleoside triphosphates at their sites of utilization [35]. Small stretches of sequence similarity were identified for different domains that might represent residues involved in nucleotide binding or catalysis (Figure 6) given the analogous reactions catalysed by both proteins. We ignore the functional significance of the differences in the *Leishmania* sequence with respect to other eukaryotic dUTPase genes, although it is possible that the enzyme exhibits features unique to protozoa. These differences could be exploited in the design of specific inhibitors against the *Leishmania* enzyme. Because dUTPase is involved in the thymidylate biosynthetic pathway and the inhibition of synthesis of dTTP has been the basis of the development of new anti-protozoan compounds, it is conceivable that the inhibition of this enzyme might represent a new avenue for rational drug design. Zalud et al. [15] have demonstrated a selective cytotoxic effect of dUTPase inhibitors on human lymphoma cells compared with fibroblasts, indicating that they act selectively on proliferating cell types. This observation, together with the fact that the sequence of the *Leishmania* enzyme is markedly different from human dUTPase, might constitute a basis for selective inhibition. The development of a high-level expression system provides a source of pure enzyme for future studies on drug design based on molecular structure.

The presence of dUTPase in a wide variety of organisms suggests that the enzyme provides a critical function in DNA replication and is essential for viability in certain organisms such as *E. coli* and yeast. In lentivirus, although dUTPase deficient (DU^-) mutants are viable, in a mouse model of infection, deletion mutants of HSV-1 were less neurovirulent, less neuroinvasive and less able to reactivate from latency than wild-type HSV-1 [36]. Recent studies have demonstrated that dUTPase is important for the efficient replication of equine infectious anaemia virus in macrophages [37]. The general idea is that DU^- viruses can replicate efficiently in actively dividing cells but fail to grow productively in non-dividing cells because dUTPase function can be borrowed from the host cell when in sufficient quantity, as occurs in actively dividing cells. It would be interesting to analyse whether infected cells exhibit alterations in dUTPase activity or whether any interaction exists between parasite and host cell activities. Similarly the full biological significance of dUTPase can now be addressed by gene replacement experiments that would establish the importance of the enzyme in parasite survival and its utility as a drug target in *Leishmania*.

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REFERENCES

1 Warner, H. R., Duncan, B. K., Garret, C. and Neuhard, J. (1981) J. Bacteriol. **145**, 687–695
2 Richards, R. G., Sowers, L. C., Laszlo, J. and Sedwick, W. D. (1986) Adv. Enzyme Regul. **22**, 157–185
3 El-Hajj, H. H., Zhang, H. and Weiss, B. (1988) J. Bacteriol. **170**, 1069–1075
4 Gadsden, M. H., McIntosh, E. M., Game, J. C., Wilson, P. J. and Haynes, R. H. (1993) EMBO J. **12**, 4425–4431
5 Strahler, J. R., Zhu, X., Hora, N., Wang, K. Y., Andrews, P. C., Roseman, N. A., Neel, J. V., Turka, L. and Hanash, S. M. (1993) Proc. Natl Acad. Sci. U.S.A. **90**, 4991–4995
6 Ladner, R. D., McNulty, D. E., Carr, S. A., Roberts, G. D. and Caradonna, S. J. (1996) J. Biol. Chem. **271**, 7745–7751
7 Ladner, R. D., Carr, S. A., Huddleston, M. J., McNulty, D. E. and Caradonna, S. J. (1996) J. Biol. Chem. **271**, 7752–7757
8 Pri-Hadash, A., Hareven, D. and Lifschitz, E. (1992) Plant Cell **4**, 149–159
9 Nation, M. D., Guzder, S. N., Giroir, L. E. and Deutsch, W. A. (1989) Biochem J. **259**, 593–596
10 Dudley, B., Hammond, A. and Deutsch, W. A. (1992) J. Biol. Chem. **267**, 11964–11967
11 Goulian, M., Bleile, B. and Tseng, B. Y. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1956–1960
12 Sedwick, W. D., Kutler, M. and Brown, O. E. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 917–921
13 Canman, C. E., Lawrence, T. S., Shewach, D. S., Tang, H. Y. and Maybaum, J. (1993) Cancer Res. **53**, 5219–5224
14 Canman, C. E., Radany, E. H., Parsels, L. A., Davis, M. A., Lawrence, T. S. and Maybaum, J. (1994) Cancer Res. **54**, 2296–2298
15 Zalud, P., Wachs, W. O., Nyman, P. O. and Zeppezauer, M. (1995) in Purine and Pyrimidine Metabolism in Man, vol. 8 (Sahota, A. and Taylor, M. W., eds.), pp. 135–138, Plenum Press, New York
16 Hochhauser, S. J. and Weiss, B. (1978) J. Bacteriol. **134**, 157–166
17 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1987) Current Protocols in Molecular Biology, Wiley Interscience, New York
18 Garvey, E. P. and Santi, D. V. (1986) Science **233**, 535–540
19 McGeoch, D. J. (1990) Nucleic Acids Res. **18**, 4105–4110
20 McIntosh, E., Ager, D. D., Gadsden, M. H. and Haynes, R. H. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 8020–8024
21 Lundberg, L. G., Thoresson, H., Karlstrom, O. H. and Nyman, P. O. (1983) EMBO J. **2**, 967–971
22 Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C. et al. (1984) Nature (London) **310**, 207–211
23 Greenberg, G. R. and Somerville, R. L. (1962) Biochemistry **48**, 247–257
24 Pogolotti, A. L., Danenberg, P. V. and Santi, D. V. (1986) J. Med. Chem. **29**, 478–482
25 Spithill, T. W. and Samaras, N. (1987) in Molecular Strategies of Parasitic Invasion (Agabian, N., Goodman, H. and Nogueira, N., eds.), pp. 269–278, Alan R. Liss, New York
26 Grumont, R., Sirawaraporn, W. and Santi, D. V. (1988) Biochemistry **27**, 3776–3784

27 Cowman, A. F., Morry, M. J., Biggs, B. A., Cross, G. A. and Foote, S. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9109–9113
28 Reche, P., Arrebola, R., Olmo, A., Santi, D. V., Gonzalez-Pacanowska, D. and Ruiz-Perez, L. M. (1994) Mol. Biochem. Parasitol. **65**, 247–258
29 Gamarro, F., Yu, P.-L., Zhao, J., Edman, U., Greene, P. J. and Santi, D. V. (1995) Mol. Biochem. Parasitol. **72**, 11–22
30 Papadopoulou, B., Roy, G. and Ouellette, M. (1993) Nucleic Acids Res. **21**, 4305–4312
31 Bello, A., Nare, B., Freedman, D., Hardy, L. and Beverley, S. M. (1995) Proc. Natl. Acad. Sci. U.S.A. **91**, 11442–11446
32 Cruz, A. K., Titu, R. and Beverley, S. M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 1599–1603
33 Barclay, B. J., Kunz, B. A., Little, J. G. and Haynes, R. H. (1982) Can. J. Biochem. **60**, 172–194
34 McDonald, P. M. and Mosig, G. (1984) EMBO J. **3**, 2863–2871
35 Wheeler, L. J., Ray, N. B., Ungermann, C., Hendricks, S. P., Bernard, M. A., Hanson, E. S. and Mathews, C. K. (1996) J. Biol. Chem. **271**, 11156–11162
36 Pyles, R. B., Sawtell, N. M. and Thompson, R. L. (1992) J. Virol. **66**, 6706–6713
37 Lichtenstein, D. L., Rushlow, K. E., Cook, R. F., Raabe, M. L., Swardson, C. J., Kociba, G. J., Issel, C. J. and Montelaro, R. C. (1995) J. Virol. **69**, 2881–2888

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