The NADP$^+$-linked glutamate dehydrogenase from *Trypanosoma cruzi*: sequence, genomic organization and expression

Patricia BARDERI*†, Oscar CAMPETELLA†*, Alberto Carlos C. FRASCH†*, José A. SANTOMÉ‡, Ulf HELLMAN§, Ulf PETTERSSON‖ and Juan José CAZZULO†*[1]

*Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín. Av. General Paz y Albarellos, Casilla de Correo 30, 1650 San Martín, Prov. de Buenos Aires, Argentina, †Instituto de Investigaciones Bioquímicas 'Luis F. Leloir', Fundación Campomar/ CONICET/Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, A. Machado 151, 1405 Buenos Aires, Argentina, ‡IQUIFIB (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 956, 1113 Buenos Aires, Argentina, §Ludwig Institute for Cancer Research, Uppsala Branch, Box 595, S-751 24 Uppsala, Sweden, and ‖Department of Medical Genetics, Biomedical Center, Uppsala University, Box 589, S-751 24 Uppsala, Sweden

NADP-linked glutamate dehydrogenase (NADP$^+$-GluDH, EC 1.4.1.4) has been purified to homogeneity from epimastigotes of *Trypanosoma cruzi* by an improved procedure, and the amino acid sequences of 11 internal peptides obtained by digestion with trypsin, endopeptidase Lys-C, endopeptidase Arg-C or CNBr have been obtained. Using oligonucleotide primers synthesized according to the amino acid sequence of the N-terminus of the mature enzyme and to the nucleotide sequence of a clone corresponding to the C-terminus, obtained by immunological screening of an expression library, two complete open reading frames ($TcGluDH_1$ and $TcGluDH_2$) were isolated and sequenced. The sequences obtained are most similar to that of the NADP$^+$-GluDH of *Escherichia coli* (70–72 % identity), and less similar (50–56 %) to those of lower eukaryotes. Using $TcGluDH_1$ as a probe, evidence for the presence of several genes and developmental regulation of the expression of NADP$^+$-GluDH in different parasite stages was obtained. $TcGluDH_1$ encodes an enzymically active protein, since its expression in *E. coli* resulted in the production of a GluDH activity with kinetic parameters similar to those of the natural enzyme.

INTRODUCTION

Glutamate dehydrogenases (GluDHs) are essential enzymes for the metabolism of amino nitrogen in most organisms, from bacteria to mammals. They act in conjunction with aminotransferases in the liberation of amino nitrogen as ammonia during amino acid catabolism, or in the fixation of ammonia for amino acid biosynthesis in plants and bacteria [1]. There are three major classes of GluDHs: one present in higher animals, which is able to use as coenzyme either NAD(H) or NADP(H) [NAD(P)$^+$-GluDHs, EC 1.4.1.3], and two others present in plants, fungi, some protozoa and bacteria, which are coenzyme-specific, strictly requiring NAD(H) (NAD$^+$-GluDHs, EC 1.4.1.2) or NADP(H) (NADP$^+$-GluDHs, EC 1.4.1.4). These GluDHs differ in structure and regulatory properties; when two GluDHs with different specificity are present in the same organism, the NAD$^+$-GluDH is usually catabolic, whereas the NADP$^+$-GluDH is biosynthetic, both often being subjected to control by metabolite induction and/or repression [1].

Trypanosoma cruzi is the parasitic protozoan that causes American trypanosomiasis, Chagas disease. The parasite has a complex life cycle, involving an obligate intracellular replicative form, the amastigote, and a non-replicative one, the bloodstream trypomastigote, in the mammalian host. The major developmental forms present in the insect vector are also a replicative stage, the epimastigote, and a non-replicative one, the infective metacyclic trypomastigote. The epimastigotes contain NAD$^+$- and NADP$^+$-GluDHs [2,3]. Both enzymes have been purified to homogeneity [4,5], and their properties studied in some detail [6,7]. The NADP$^+$-GluDH, which is a hexamer made up of identical subunits with an apparent M_r of 47000, seems to be similar to the enzyme from *Escherichia coli* in terms of amino acid composition and N-terminal sequence [8]. Although subcellular fractionation experiments suggest that the NADP$^+$-GluDH might have a double subcellular localization, with a small mitochondrial component [9], immunoelectron microscopic evidence suggests that the enzyme is cytosolic [10]. The role of the NADP$^+$-GluDH is not clear, although it has been suggested that it might be biosynthetic [11]. The NAD$^+$-GluDH, which is largely mitochondrial, seems to have a catabolic role in the parasite, since it increases in the late exponential phase of growth, when amino acids are certainly being consumed and NH_3 is produced [12], and is strongly inhibited by ATP [3,5,7] and by acetyl-CoA [7]. We have proposed that both GluDHs might be involved in the reoxidation of glycolytic NADH through L-alanine production and excretion [9]. A similar role has been postulated for the NADP$^+$-GluDH from the anaerobic protozoan *Giardia intestinalis* [13].

Although GluDHs have been sequenced in a number of organisms, the only sequence of such an enzyme available from a Protozoan is that from the primitive eukaryote *G. intestinalis*, which has high similarity with the *E. coli* and *Neurospora crassa* enzymes [13]. Since Trypanosomatids are also ancient organisms, but their enzymes sequenced so far have been shown in some cases to be most similar to those of plants or mammals, it was considered interesting to add the NADP$^+$-GluDH from *T. cruzi* to these comparisons. In addition, cloning and sequencing the enzyme would allow us to determine the possible presence of different genes encoding enzyme isoforms targeted to different subcellular compartments. The study of the expression of the NADP$^+$-GluDH in different parasite stages, which live in quite different nutritional environments, also seemed likely to be rewarding. We report here the purification, cloning, sequencing and determination of the genomic organization of the NADP$^+$-

Abbreviations used: GluDH, glutamate dehydrogenase; PFGE, pulsed-field gel electrophoresis; TFA, trifluoroacetic acid; IPTG, isopropyl β-D-thiogalactoside; RT, reverse transcriptase.

[1] To whom correspondence should be addressed at: Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín. Av. General Paz y Albarellos, Casilla de Correo 30, 1650 San Martín, Prov. de Buenos Aires, Argentina.

GluDH from *T. cruzi*, and its expression in different parasite stages, as well as in *E. coli*, as a recombinant protein. We show that the enzyme is most similar to the *E. coli* enzyme, probably has an exclusively cytosolic localization and is expressed in all parasite stages, being more abundant in the insect stage, which must rely upon amino acids as the major carbon and energy source.

MATERIALS AND METHODS

Organism and culture

Epimastigotes of *T. cruzi* were grown and harvested as previously described [12]. The Tul2 strain was used for enzyme purification; the cloned stocks Tul10, Sylvio X10/7, HO3/5, CanIII, NIH, YIE9, YIE10, CA-I/72 and CA-I/72hdna, provided by Dr. J. A. Dvorak, NIAID, NIH, Bethesda, MD, U.S.A., were used for pulsed-field gel electrophoresis (PFGE) experiments. Amastigotes and trypomastigotes were obtained by infection of Vero cell monolayers with trypomastigotes of the RA strain [14]. Trypomastigotes were obtained free of cellular debris by leaving them to swim off the centrifuged pellet by incubating for 1 h at 37 °C [14]. Amastigotes were contaminated with debris to some extent, but control experiments showed that the contaminating material did not interfere with the enzyme assays. The cells were washed three times in a solution containing 0.25 M sucrose and 5 mM KCl, and the cell-free extract was obtained after cell disruption by three cycles of freezing and thawing, as previously described [15].

Purification of the enzyme

The cell-free extract was submitted to ammonium sulphate fractionation and DEAE-cellulose chromatography, as previously described [8]. The Sephadex G-200 gel-filtration step was replaced by affinity chromatography performed on a Blue Sepharose (Pharmacia Biotech, Uppsala, Sweden) column (1.5×15 cm) equilibrated with 50 mM Tris/HCl buffer, pH 7.6, containing 50 mM NaCl (buffer 1). After exhaustive washing of the column with the same solution, down to negligible absorbance at 280 nm, the enzyme was eluted as a sharp peak by the addition of 2 mM $NADP^+$ in buffer 1. The active fractions were pooled, dialysed against buffer 1, and used for the experiments described.

Determination of enzyme activity

The $NADP^+$-GluDH activity was assayed in the direction of reductive amination of α-oxoglutarate, as previously described [4]. One enzyme unit is defined as the amount of enzyme leading to the reduction of 1 μmol of $NADP^+$ per min at 25 °C. Apparent kinetic constants were determined with a computer program fitting the data to a hyperbola by applying the Gauss–Newton algorithm [16].

Protein determination

Protein content was determined in cell-free extracts by the method of Bradford [17].

PAGE

Enzyme samples were subjected to PAGE in the presence of SDS at room temperature in slab gels (10 % acrylamide) under reducing conditions, as described by Laemmli [18]. The gels were stained for protein with Coomassie Brilliant Blue R-250. The fragments obtained by CNBr digestion were separated by SDS/PAGE in a high-density gel run in the Phast System™ (Pharmacia Biotech, Uppsala, Sweden).

Immunoblotting

The proteins separated on SDS/PAGE gels, run as described above, were electroblotted onto nitrocellulose sheets, processed and incubated with a monospecific polyclonal serum, at a dilution of 1:1500, and the blots were developed as previously described [19]. The antiserum against purified *T. cruzi* $NADP^+$-GluDH was obtained as described [20].

Reduction and pyridylethylation of the $NADP^+$-GluDH

The sample (approx. 1.4 mg of purified $NADP^+$-GluDH), dried and re-dissolved in a solution containing 6 M guanidine/HCl, 0.25 M Tris/HCl, pH 8.5, and 1 mM EDTA, was reduced and pyridylethylated [21], and then desalted by HPLC on a 250×4.6 mm Vydac C_4 column, by using a 0–80 % (v/v) acetonitrile gradient in 0.1 % (v/v) trifluoroacetic acid (TFA), at a flow rate of 1 ml/min.

CNBr cleavage

The reduced and pyridylethylated sample (200 μg) was dried and dissolved in 70 % (v/v) formic acid. CNBr (0.1 mg) was added, also as a solution in 70 % (v/v) formic acid, and the mixture was incubated at room temperature in the dark for 24 h. The sample was then diluted with water and taken to dryness in a Speed Vac rotatory desiccator (Savant Instruments). The fragments obtained were separated by SDS/PAGE as described above, and transferred to a PVDF membrane (Pro-Blott™) for sequencing.

Tryptic digestion

Approximately 100 μg of the reduced and pyridylethylated $NADP^+$-GluDH were dialysed overnight at 4 °C against 0.1 M ammonium bicarbonate. Digestion was performed in the same buffer solution in the presence of 2 M urea, at a trypsin:$NADP^+$-GluDH ratio of 1:25, for 48 h at 37 °C. After centrifugation for 10 min at 8000 ***g*** in an Eppendorf microcentrifuge, the supernatant was acidified with TFA and the peptides were separated by HPLC on a 250×4.6 mm Vydac C_{18} column, by using a 0–70 % acetonitrile gradient in 0.1 % TFA, at a flow rate of 0.8 ml/min.

Digestion with endopeptidases Lys-C and Arg-C

Digestion with endopeptidases Lys-C and Arg-C was performed as described above for trypsin, also using 100 μg of the reduced and pyridylethylated $NADP^+$-GluDH in each case, except for the time (24 h for Lys-C, 6–8 h for Arg-C) and the proteinase/$NADP^+$-GluDH ratio, which was 1:100. Peptide purification was also as described above for trypsin digestion.

Peptide sequencing

The selected CNBr fragments, blotted onto a PVDF membrane, as well as selected peptides obtained after digestion with trypsin, endopeptidase Lys-C or endopeptidase Arg-C and applied to a polybrene-coated glass filter, were sequenced in an Applied Biosystems model 477 automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.), run according to the manufacturer's instructions.

DNA and RNA purification

Genomic DNA [22] and total RNA [23] were obtained from the parasites as described in the respective references.

Immunological screening of a genomic library of *T. cruzi*

An expression genomic library of *T. cruzi*, Miranda strain, made in phage λgt11 [24], was screened using the polyclonal serum anti-NADP$^+$-GluDH, by conventional procedures [22].

Southern and Northern blots

Southern and Northern blots were performed using standard procedures [22]; hybridization to nylon filters (Amersham Hybond-N) was done with probes labelled with [^{32}P]dCTP (Du Pont) by random priming [22].

PFGE

The samples were prepared [25] and PFGE was performed in a Bio-Rad CHEF Mapper apparatus [26], as previously described. The DNA was nicked by HCl treatment, transferred to nylon filters and used for hybridizations as described in the Southern and Northern blots section above.

PCR and cloning procedure of the gene

Two primers were designed following the N-terminus sequence of the NADP$^+$-GluDH [8] and the nucleotide sequence corresponding to the 3′-end of the gene contained in the cloned fragment (see the Results section). The sequences of the primers were: N-ter 5′ TATCATATGAARTAYACIAGYGTIGAYG-AYTTYATHAA 3′ and C-ter 5′ TTTCAATGCTTCCCAAC-AGAGAT 3′, where I, Y, R and H correspond to inosine, T or C, A or G and T, C or A, respectively.

The PCR reaction was carried out with 500 ng of *T. cruzi* DNA or DNA from a *T. cruzi* library constructed in the phage λgt11 [24] in a Perkin-Elmer 480 thermocycler using 40 cycles under the following conditions: 94 °C 4 min, 93 °C 0.5 min, 62 °C 0.5 min, 72 °C 1 min. The final extension was performed at 72 °C for 10 min. The PCR products were cloned in a pT7 Blue T-Vector (Novagen) and were used for sequencing and as a probe in Southern and Northern blots.

In order to obtain the untranslated 5′-end region of the mRNA, reverse transcriptase (RT)-PCR was performed on 2–5 μg of total RNA purified from epimastigotes of the Tul 2 strain with Superscript RT Plus™ (Life Technologies Inc., Gaithesburg, MD, U.S.A.). The oligonucleotide primers used were: one corresponding to the mini-exon which is added post-translationally to all mRNAs in *T. cruzi* [27], with the sequence 5′ AACTAACGGTATTATTGATA 3′, and two primers from internal coding sequences of the $TcGluDH_1$ gene, namely Int_1: 5′ ATTGGGGTCGCGCTTCTTCAC 3′ and Int_2: 5′ TTCAC-CCTTGTCATCCACCCA 3′.

Hemi-nested PCR was performed using the mini-exon and Int_2 primers in the first reaction, followed by a second reaction using the mini-exon and the Int_1 primers. The conditions used for the first PCR reaction were denaturation for 5 min at 93 °C and 35 cycles under the following conditions: 93 °C for 0.5 min; 40 °C for 0.5 min; 72 °C for 1 min. The final extension was performed at 72 °C for 10 min. The conditions used for the second PCR reaction were similar, except that 20 cycles were performed, and annealing was at 47 °C.

For the expression of the recombinant enzyme in *E. coli*, the complete $TcGluDH_1$ gene was amplified by PCR using oligonucleotide primers corresponding to the 5′- and 3′-ends of the cloned gene, with the addition of sequences containing the *Nde*I and *Xho*I sites respectively, as follows: Exp_1 5′ TATACATAT-GAAGTACACGAGCGTGG 3′; Exp_2 5′ ATGAAGGGTCT-TGGCGTAGTTCTCGAGCAC 3′.

The PCR reaction was run under the same conditions described above for the primers N-ter and C-ter.

Sequencing of DNA

Sequence determination was performed by the dideoxy chain-termination method [28] using the Sequenase version 2.0 DNA Sequencing Kit (U.S. Biochemical Inc.). The selected clones obtained by PCR amplification ($TcGluDH_1$ and $TcGluDH_2$) were completely sequenced with the help of internal primers. All sequences were completed on both DNA strands.

Expression of active recombinant NADP$^+$-GluDH

The $TcGluDH_1$ gene was expressed in the plasmid vector pET in *E. coli* BL26 cells [29]. Bacterial cultures (100 ml), when they reached an A_{600} of 0.6, were induced with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) at 28 °C, harvested at an absorbance close to 4.0, and used for the purification of recombinant enzyme. For purification, bacteria were lysed for 20 min at room temperature in a solution containing 50 mM Tris/HCl buffer, pH 7.6, 150 mM NaCl, 1 mM PMSF, lysozyme (100 μg/ml) and 1% Triton X-100. After lysis, 10 mM $MgCl_2$ and DNAase (20 μg/ml) were added, and the homogenate was incubated for 15 min at room temperature and afterwards centrifuged for 15 min at 20000 ***g*** at 4 °C. The cell-free extract was dialysed against buffer 1 and applied to a Blue Sepharose column, run as described for the purification of the natural enzyme.

Chemicals

DEAE-Sephacel and Blue Sepharose were obtained from Pharmacia Biotech, Uppsala, Sweden. Molecular-mass markers and enzyme substrates were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Reagents for peptide sequencing were obtained from Applied Biosystems, Foster City, CA, U.S.A. All other chemicals were analytical grade reagents.

RESULTS

Purification and partial amino acid sequencing of the NADP$^+$-GluDH

Table 1 shows the results of a typical purification of the NADP$^+$-GluDH from *T. cruzi* epimastigotes, Tul 2 strain. The introduction of an affinity chromatography step with Blue Sepharose, instead of the gel-filtration step used before [8], allowed a better purification, resulting in a higher specific activity (330 units/mg, as compared with 189 units/mg reported in [8]) and a larger yield. When the different fractions in the purification procedure were submitted to SDS/PAGE, the Blue Sepharose eluate from overloaded gels presented only traces of proteins other than the 47 kDa NADP$^+$-GluDH subunit (results not shown).

Peptides were generated from the purified NADP$^+$-GluDH by digestion with trypsin, endopeptidase Lys-C or endopeptidase Arg-C, and purified by reverse-phase HPLC, as described in the Materials and methods section. CNBr peptides were purified by SDS/PAGE, blotted onto PVDF membranes, and their N-termini were sequenced, as described in the Materials and methods section. The sequences obtained were FTGK, CLQ-LGAK and TNYV (trypsin); NSLTTLPMGGGK, HGTVE-EYAK, YGTEGGK and VAEAMK (Lys C); HLGADTD (Arg C); and RPEATGYGLVYFLDALLKKHNQELXG, DIKNV-KHGTVEEYAKGLG and SQNAARL (CNBr). These peptides, which together with the N-terminal sequence of the GluDH

Table 1 Purification of the NADP$^+$-GluDH from *T. cruzi*

The enzyme was purified from 21 g (wet weight) of epimastigotes, as described in the Materials and methods section.

Step	Volume (ml)	Protein (mg)	Enzyme activity		Purification (-fold)	Yield (%)
			Total (units)	Specific (units/mg)		
Cell-free extract	106	1160	6540	5.6	1	100
Ammonium sulphate fractionation (50–75%)	25	250	5840	23.3	4.2	89
DEAE-cellulose chromatography	28	29	5040	174	31	77
Blue-Sepharose affinity chromatography	2.3	11.5	3800	330	59	58

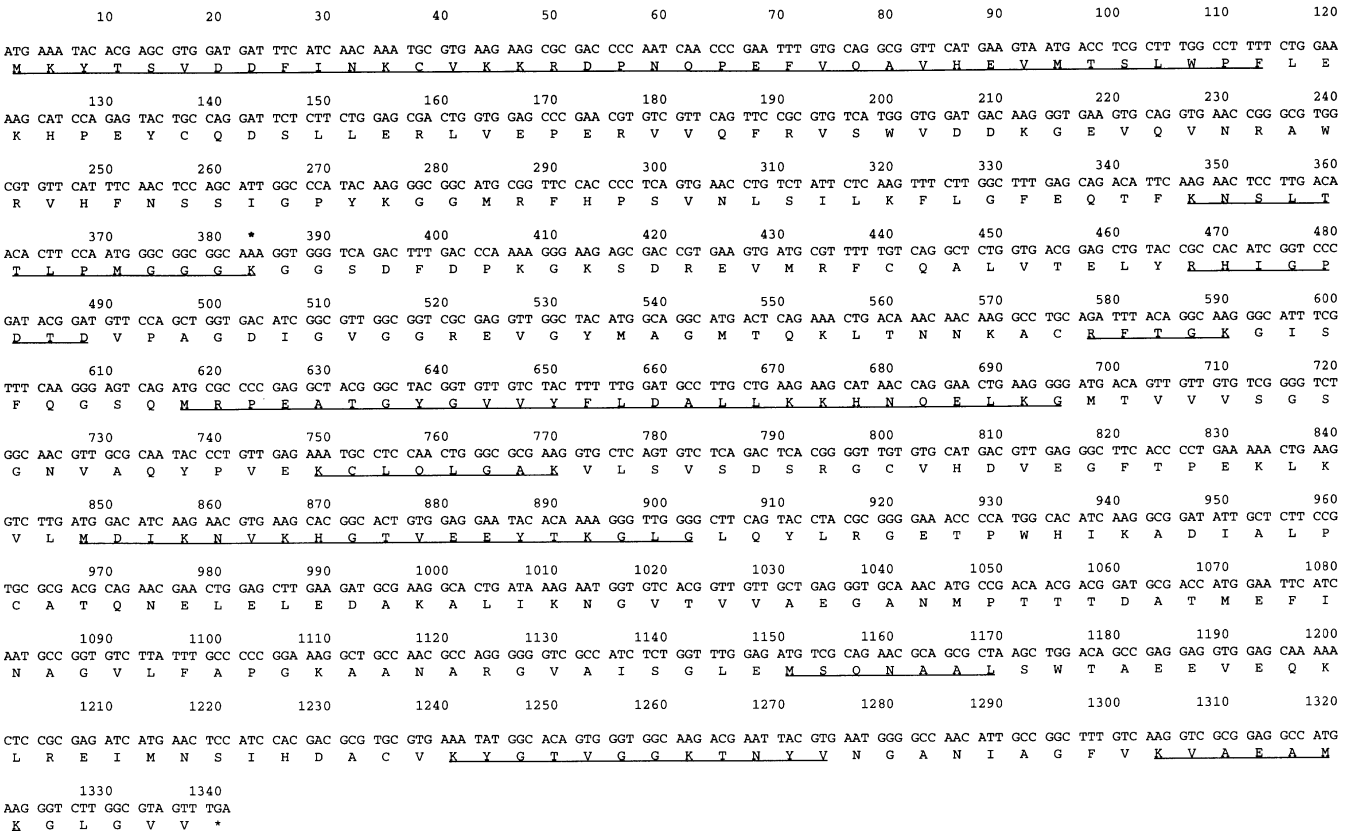

Figure 1 Sequence of the *TcGluDH$_2$* clone encoding the NADP$^+$-GluDH from *T. cruzi*

The full sequence was obtained from DNA sequencing (GenBank accession number AF009365); the underlined stretches were confirmed by sequencing peptides isolated after digestion with trypsin, endopeptidase Arg-C, endopeptidase Lys-C or CNBr. The N-terminus had been directly sequenced previously [8]. The active site Lys residue is indicated by an asterisk.

previously determined [8] account for close to one-third of the molecule, are underlined in the full sequence deduced from sequencing of the *TcGluDH$_2$* clone, shown in Figure 1. A few discrepancies were found between sequences obtained by peptide sequencing and those obtained from DNA sequencing (see below), and they are indicated by underlining in the sequences above.

Cloning, sequencing and genomic organization of genes encoding the NADP$^+$-GDH from *T. cruzi*

Two complete coding sequences for the NADP$^+$-GluDH, *TcGluDH$_1$* and *TcGluDH$_2$*, were identified by PCR in genomic DNA from two parasite stocks, M76 and Tul2 respectively (GenBank accession numbers AF009364 and AF009365). The 5′-end primer was designed according to the information obtained from direct protein sequencing [8]. The sequence for the 3′-end primer was obtained from a clone identified in a phage λgt11 expression genomic library of *T. cruzi* after screening with a polyclonal monospecific anti-NADP$^+$-GluDH serum (results not shown). The complete open reading frame consists in both cases of 1338 nucleotides, encoding a protein made up of 446 amino acid residues. Figure 1 shows the nucleotide sequence of clone *TcGluDH$_2$*, as well as the deduced protein sequence. The TcGluDH$_1$ and the TcGluDH$_2$ proteins had 96% overall identity (not shown).

Since the N-ter primer used for amplification of the genes was derived from the sequence of the N-terminus of the mature

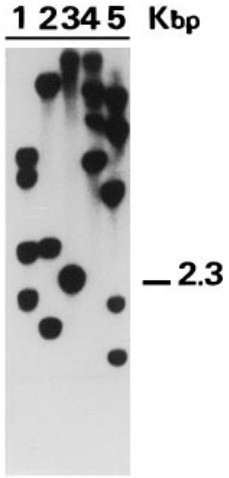

Figure 2 Southern blot of genomic DNA (*T. cruzi*, Tul2 strain) digested with different restriction enzymes

The enzymes used were *Eco*RI (lane 1); *Hin*dIII (lane 2); *Bgl*II (lane 3); *Ssp*I (lane 4) and *Pst*I (lane 5). The complete *TcGluDH*$_1$ clone was used as probe.

enzyme, the possible presence of two genes, encoding isoforms with different subcellular localization and differing in the presence or absence of a mitochondrial transit peptide, could not be excluded. In order to investigate further this possiblity, heminested RT-PCR was performed on Tul 2 total RNAs as described in the Materials and methods section, using as primers oligonucleotides corresponding to the sequence of the mini-exon and two internal sequences of *TcGluDH*$_1$. Only one fragment of 139 bp in length was obtained. Two clones were partially sequenced. They consisted of the mini-exon (39 bps), a 5′-untranslated region of 40 bps (AAAACTAATTTTAAAGAA-AAGGAAAAACAAATACATCATA), and 60 bps corresponding to the N-terminus of GluDH. These results make unlikely the possible presence of two different genes, one of them encoding a mitochondrial transit peptide.

The *TcGluDH*$_1$ clone was used as probe for Southern blots. Figure 2 shows that when genomic DNA from the parasite was cut with several restriction enzymes, a pattern suggesting the presence of more than a single gene per haploid genome was found. Partial digestions (0–90 min) with *Bgl*II, a restriction enzyme cutting immediately after the stop codon, suggested that these genes are not arrayed in tandem (not shown), as is frequently the case in Trypanosomatids. Further evidence suggesting that there are several genes encoding NADP$^+$-GluDH in *T. cruzi* came from sequence analysis (Figure 3). Comparison of the 5′-end sequence from three genomic clones of M76 and three clones (1 genomic and 2 cDNA) from Tul2 stocks showed that all of them have some conservative nucleotide changes. These results suggest that there are at least three different GluDH genes in both parasite strains, in agreement with the results obtained by Southern blots. Although errors introduced by PCR cannot be excluded, the conservative nature of all the changes detected makes this alternative rather unlikely.

A Southern blot of PFGE-resolved parasite chromosomal bands, probed with the *TcGluDH*$_1$ clone, revealed two hybridization bands (not shown) in the strains and cloned stocks of the parasites listed in the Materials and methods section. One of these bands corresponded to the compression zone, which under this particular set of PFGE conditions contains chromosomes of size bigger than 2500 kbps, and the other band was found between 1035 and 1580 kbp, in the different parasite stocks.

Expression of the NADP$^+$-GluDH in different developmental stages of *T. cruzi*

Northern blot experiments, using total parasite RNA hybridized with the *TcGluDH*$_1$ clone, demonstrated that expression of NADP$^+$-GluDH is developmentally regulated. Whereas a strong band of about 2.3 kb was observed in the epimastigote stage, even after a long period of exposure the X-ray film showed only very weak spots (barely visible in Figure 4) in the case of amastigotes and trypomastigotes. In good agreement with this result, the Western blot in Figure 4(B) shows a very strong reaction with the epimastigote extracts, with weak reactions for the extracts from amastigotes and trypomastigotes. Direct determination of the activity of NADP$^+$-GluDH in these extracts gave the following specific activity values (averages of duplicates not differing by more than 10 %, expressed as units · min^{-1} · mg of protein^{-1}): 11.6 (epimastigotes, Tul2 strain), 11.4 (epimastigotes, RA strain), 0.87 (trypomastigotes, RA strain) and 0.05 (amastigotes, RA strain).

Expression of a recombinant NADP$^+$-GluDH from *T. cruzi* in *E. coli*

E. coli BL26 cells, transformed with the *TcGluDH*$_1$ gene cloned in pET, were grown and lysed, and the recombinant protein was

```
ATGAAGTACACGAGCGTGGATGACTTTATCAACAAGTGCG N-ter T. cruzi GluDH 1
ATGAAGTATACGAGTGTGGACGATTTTATTAACAAGTGCG N-ter phage 1
ATGAAATACACGAGCGTGGACGATTTCATTAACAAGTGCG N-ter phage 2
ATGAAATACACGAGCGTGGATGATTTCATCAACAAATGCG N-ter T. cruzi GluDH 2
ATGAAGTACACTTCTGTGGACGACTTTATCAACAATGCG N-ter 1
ATGAAGTACACTTCCGTGGACGACTTTATCAACAAGTGCG N-ter 2

TGAAGAAGCGCGACCCCAAT                      N-ter T. cruzi GluDH 1
TGAAGAAGCGCGACCCCAAT                      N-ter phage 1
TGAAGAAGCGCGACCCCAAT                      N-ter phage 2
TGAAGAAGCGCGACCCCAAT                      N-ter T. cruzi GluDH 2
TGAAGAAGCGCGACCCCAAT                      N-ter 1
TGAAGAAGCGCGACCCCAAT                      N-ter 2
```

Figure 3 Microheterogeneities in the nucleotide sequence at the 5′-end of the coding sequence of the NADP$^+$-GluDH gene

Two fully sequenced clones (*TcGluDH*$_1$ and *TcGluDH*$_2$) and four partially sequenced clones (two from recombinant phages, N-ter phage 1 and 2, and and two from genomic DNA, N-ter 1 and 2) are compared. Differences in nucleotide sequence are boxed.

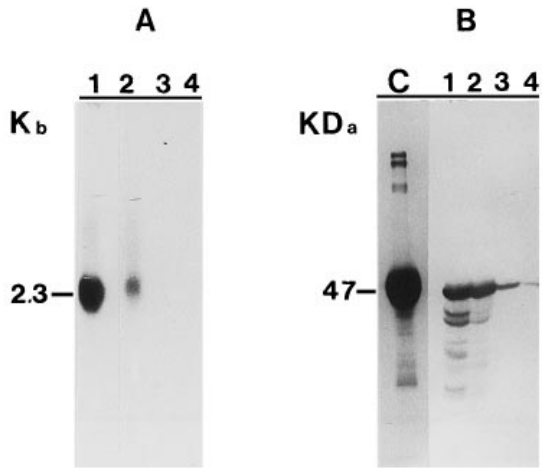

Figure 4 Expression of the NADP$^+$-GluDH in different developmental stages of the parasite

(**A**) Northern blot: lane 1, epimastigotes of the Tul2 strain; lanes 2, 3 and 4, epimastigotes, trypomastigotes and amastigotes respectively of the RA strain. Total parasite RNA (30 μg) was used per lane. The complete *TcGluDH*$_1$ clone was used as probe. (**B**) Western blot: lane numbers as in (**A**); lane C, purified NADP$^+$-GluDH as control. Total parasite proteins or purified enzyme (50 or 1 μg of protein respectively) were used in each lane. The blot was reacted with a monospecific polyclonal antiserum against purified GluDH.

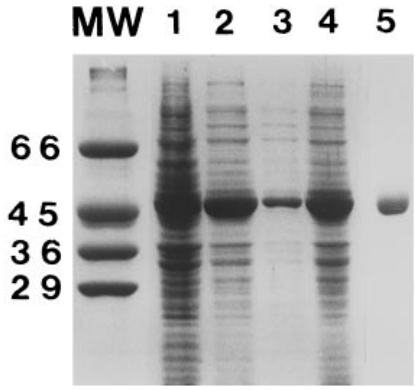

Figure 5 Expression of the NADP$^+$-GluDH from *T. cruzi* in *E. coli*

MW, molecular-mass markers; lanes 1 and 2, total proteins from a non-induced and an induced cultures respectively; lanes 3 and 4, soluble and insoluble proteins from the induced culture respectively; lane 5, NADP$^+$-GluDH eluted from Blue Sepharose with 2 mM NADP.

purified, as described in the Materials and methods section. As shown in Figure 5, the IPTG-induced cells presented, when run on SDS/PAGE, a strong Coomassie Blue-stained band of 47 kDa, which was found both in the soluble extract and in the pellet. In excellent agreement, direct determination of NADP$^+$-GluDH activity indicated that, whereas the enzyme activity in IPTG-induced *E. coli* cells containing an unrelated plasmid was very low (0.20 units$\cdot$min$^{-1}\cdot$mg of protein^{-1}), the non-induced BL26-1 cells presented a higher activity (8.78 units$\cdot$min$^{-1}\cdot$mg of protein^{-1}) and the induced cells showed an activity (108.33 units$\cdot$min$^{-1}\cdot$mg of protein^{-1}) that was 542-fold higher than that of the induced unrelated control. The recombinant TcGluDH$_1$ enzyme was purified by a Blue-Sepharose affinity chromatography step identical with that used for the purification of the natural enzyme from epimastigotes, which yielded homogeneous recombinant NADP$^+$-GluDH in a single step (Figure 5). This enzyme presented apparent K_m values for the substrates in the direct and reverse reactions that were not significantly different from those shown by the natural enzyme (Table 2).

Table 2 Apparent K_m values for substrates in the direct and reverse reactions catalysed by the NADP$^+$-GluDH from *T. cruzi*

Substrate	Apparant K_m (mM)	
	Natural enzyme	Recombinant enzyme
α-Oxoglutarate reductive amination		
α-Oxoglutarate	1.27 $\pm$ 0.45	0.84 $\pm$ 0.3
NH_4Cl	2.03 $\pm$ 0.36	3.30 $\pm$ 0.52
NADPH	0.013 $\pm$ 0.0015	0.010 $\pm$ 0.0005
L-Glutamate oxidative deamination		
L-Glutamate	2.10 $\pm$ 0.25	0.90 $\pm$ 0.63
NADP	0.0043 $\pm$ 0.0005	0.0069 $\pm$ 0.001

DISCUSSION

The modified purification procedure described here, as compared with the previously published one [8], allowed us to obtain NADP$^+$-GluDH preparations of higher specific activity (probably due to the absence of denatured enzyme, which co-purifies with the native enzyme in conventional steps, but does not bind to Blue Sepharose in the affinity chromatography step) and with a higher yield. Some lower-molecular-mass protein traces, apparent in overloaded SDS/PAGE gels, copurified with the enzyme in Superose 6 gel filtration in an FPLC system and cross-reacted antigenically with a polyclonal monospecific anti-GluDH serum (results not shown); they are likely, therefore, to be minor products of proteolytic degradation of the NADP$^+$-GluDH. The purified enzyme is very stable, and can be kept for many months at 4 °C with little inactivation. After long-term storage, some preparations (such as the one used as control in Figure 4B) presented small amounts of high-molecular-mass material, probably due to enzyme aggregation.

The complete sequence of the enzyme was obtained by a combination of protein chemistry and molecular biology approaches. The sequenced peptides, amounting to about one-third of the complete molecule, matched well the full sequence deduced from sequencing *TcGluDH*$_2$, cloned from the same parasite strain (Tul2).

The NADP$^+$-GluDH seems to be encoded by at least two, and probably more, genes in the parasite's genome, as suggested by the Southern blots and by the heterogeneities found in the nucleotide sequences at the 5′-end of the coding region. At variance with a number of cases that have been well studied in *T. cruzi*, like those of cruzipain [30] and tyrosine aminotransferase [31], no evidence of a tandem arrangement was found. Although biochemical evidence had previously suggested that the enzyme has a multiple localization, most of it being cytosolic, but with a secondary mitochondrial localization [9], the evidence presented here casts doubts on the latter, and supports the evidence obtained by immunoelectron microscopy [10], which suggests an exclusively cytosolic localization for NADP-GluDH. A gene coding for a mitochondrial isoform, at variance with the one encoding the cytosolic enzyme, should have a transit peptide; the PCR studies reported herein, using as primers the sequence of the mini exon and internal sequences of the *TcGluDH*$_1$ gene, showed no evidence for such a peptide, and the N-terminal Met residue

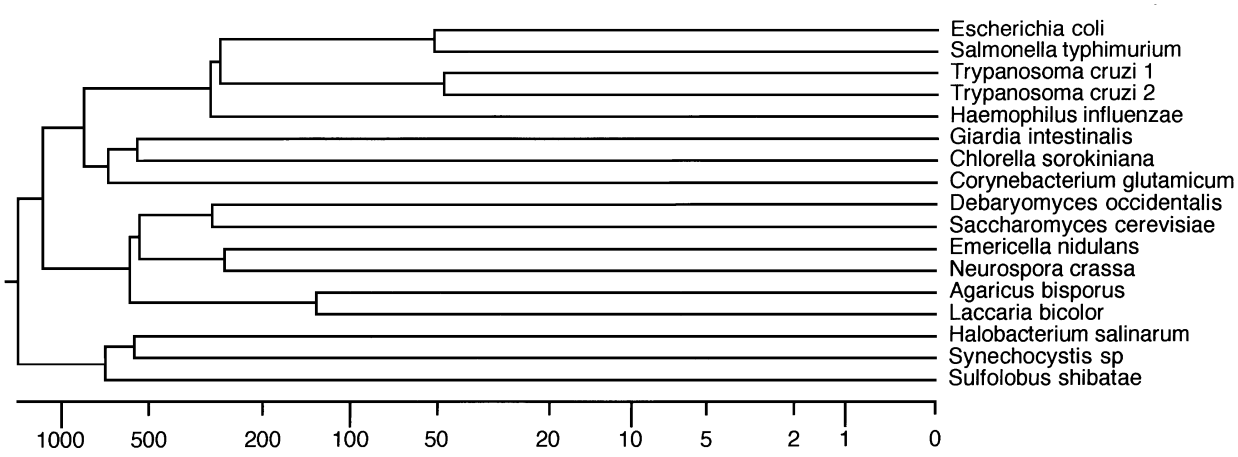

Figure 6 Phylogenetic tree of NADP$^+$-GluDH

The sequence of TcGluDH$_2$ was compared with those of TcGluDH$_1$ and the NADP-GluDHs from *E. coli* [32], *S. typhimurium* [33]. *H. influenzae* [34], *G. intestinalis* [13], *C. sorokiniana*, [36], *Corynebacterium glutamicum* [43], *Debaryomyces occidentalis* [44], *S. cerevisiae* [38], *Emericella nidulans* [45], *N. crassa* [37], *Agaricus bisporus* [46], *Laccaria bicolor* (Swiss-Prot accession number P54388), *H. salinarium* [39], *Synechocystis* sp. [47] and *S. shibatae* [40], using the Multiple Sequence Alignment Programme Megalign, from Lasergene (DNASTAR Inc., Madison, WI, U.S.A.), which employs the algorithm of Hein [48].

of the mature protein was the only possible initiation Met residue found. Southern blots obtained after PFGE showed, in several cloned stocks of the parasite, hybridization of the full *TcGluDH*$_1$ gene used as a probe with chromosomes of considerable difference in size.

Sequence comparisons showed that the *T. cruzi* NADP$^+$-GluDH encoded by the cloned genes is far more related to the eubacterial enzymes from *E. coli* [32], *Salmonella typhimurium* [33] and *Haemophilus influenzae* [34] than to those from lower eukaryotes, such as another protozoan, *G. intestinalis* [13] (supposed to be the oldest eukaryote [35]), the unicellular alga *Chlorella sorokiniana* [36] and fungi such as *N. crassa* [37] and *Saccharomyces cerevisiae* [38]. Figure 6 shows the relationships among the sequences of these NADP$^+$-GluDHs. Taking TcGluDH$_2$ as the basis for comparison, the enzymes from *E. coli*, *S. typhimurium* and *H. influenzae* had 70, 70 and 67 % identity respectively; if TcGluDH$_1$ is taken for this comparison, the identity with the bacterial enzymes is slightly higher, 72, 71 and 68 % respectively. The identity of TcGluDH$_2$ with the enzymes from lower eukaryotes was between 56 % for *G. intestinalis* and 50 % for *S. cerevisiae*. The lowest identity was found when the *T. cruzi* NADP$^+$-GluDH sequences were compared with those from Archaebacteria, such as *Sulfolobus shibatae* [39] and *Halobacterium salinarium* [40] (29 and 28 % identity respectively). It is noteworthy that in the case of other enzymes from *T. cruzi* involved in protein and amino acid catabolism, some have been shown to be most similar to the corresponding enzymes from mammals, as in the case of tyrosine aminotransferase [31], whereas the partially sequenced alanine aminotransferase from the parasite shows the highest similarity towards the corresponding enzymes from plants [41].

The *TcGluDH*$_1$ gene encodes an enzymically active protein, as shown by expression in *E. coli* followed by purification by affinity chromatography. The highly similar NADP$^+$-GluDH in *E. coli* did not interfere with the expression of the *T. cruzi* enzyme, since the basal endogenous bacterial activity, due to its tight regulation, was very low compared with that of the recombinant enzyme. This recombinant enzyme should be useful for structural studies if it proves easy to obtain crystals with good stability and yield. In addition, since the native enzyme is highly stable, keeping its full activity for many months, even as a dilute solution at 4 °C, the possibility of high-scale production of the recombinant NADP$^+$-GluDH in *E. coli* makes feasible its use as a reagent for the enzymic determination of ammonia and as a coupled enzyme for transaminase assays.

The expression of NADP$^+$-GluDH in *T. cruzi* is developmentally regulated in a way that is reminiscent of the situation found with the major cysteine proteinase, cruzipain, from the same parasite. Namely, the axenic-culture epimastigote form presented, in both cases, considerably higher enzyme activity and level of enzyme protein, as compared with the cell-culture-derived amastigote and trypomastigote forms. However, whereas in the case of cruzipain there was no correlation between the levels of mRNA and enzyme protein, thus suggesting that the regulation of expression of the proteinase is post-transcriptional [42], in the case of the NADP$^+$-GluDH there was a much better correlation. In fact, the levels of mRNA detected in extracts from trypomastigotes and amastigotes were nearly negligible compared with those in extracts of the epimastigote form, and the protein levels were certainly much lower than in the latter form. The different levels of the NADP$^+$-GluDH in the different parasite forms suggest that the role played by the enzyme in amino acid metabolism in these forms is probably at least quantitatively different. The bloodstream trypomastigote form has the possibility of using glucose as a major carbon source; the source used by the intracellular amastigote form is not clear. On the other hand, the major insect form, the epimastigote, is likely to depend almost exclusively on amino acids as a carbon and energy source. In fact, the Triatomine insect's gut, shortly after the blood meal, should become almost completely deprived of carbohydrates, whereas the major nutrients will be the amino acids derived from haemoglobin degradation by the insect's peptidases. The much higher NADP$^+$-GluDH activity in this parasite form suggests that the enzyme might have a role connected somehow with catabolism and energy generation, despite the fact that in other, more biochemically conventional organisms, it is essentially a biosynthetic enzyme.

O.C., A.C.C.F., J.A.S. and J.J.C. are members of the Carrera del Investigador Científico from the National Research Council of Argentina (CONICET), and P.B. is

the recipient of a Research Scholarship from the same Institution. We are indebted to Ms. Berta Franke de Cazzulo for providing the parasite cultures and the PFGE Southern blots used. This work was supported by grants from CONICET and the Swedish Agency for Research Cooperation with Developing Countries (SAREC).

REFERENCES

1 Smith, E. L., Austen, B. M., Blumenthal, K. M. and Nyc, J. F. (1975) Enzymes (3rd edn.) **11**, 293–367
2 Cazzulo, J. J., Juan, S. M. and Segura, E. L. (1977) Comp. Biochem. Physiol. **56B**, 301–303
3 Cazzulo, J. J., Franke de Cazzulo, B. M., Higa, A. I. and Segura, E. L. (1979) Comp. Biochem. Physiol. **64B**, 129–131.
4 Juan, S. M., Segura, E. L. and Cazzulo, J. J. (1978) Int. J. Biochem. **9**, 395–400.
5 Walter, R. D. and Ebert, F. (1979) J. Protozool. **26**, 653–656
6 Cazzulo, J. J. (1984) Comp. Biochem. Physiol. **79B**, 309–320
7 Urbina, J. A. and Azavache, V. (1984) Mol. Biochem. Parasitol. **11**, 241–255
8 Cazzulo, J. J., Nowicki, C., Santomé, J. A., Wernstedt, C. and Hellman, U. (1988) FEMS Microbiol. Lett. **56**, 215–220
9 Duschak, V. G. and Cazzulo, J. J. (1991) FEMS Microbiol. Lett. **83**, 131–136
10 Souto-Padrón, T., Campetella, O., Cazzulo, J. J. and de Souza, W. (1990) J. Cell Sci. **96**, 485–490
11 Carneiro, M. and Caldas, R. A. (1983) Comp. Biochem. Physiol. **75B**, 61–64
12 Cazzulo, J. J., Franke de Cazzulo, B. M., Engel, J. C. and Cannata, J. J. B. (1985) Mol. Biochem. Parasitol. **16**, 329–343
13 Yee, J. and Dennis, P. P. (1992) J. Biol. Chem. **267**, 7539–7544
14 Andrews, N. W. and Colli, W. (1982) J. Protozool. **29**, 264–269
15 Cazzulo, J. J., Couso, R., Raimondi, A., Wernstedt, C. and Hellman, U. (1989) Mol. Biochem. Parasitol. **33**, 33–41
16 Fraser, R. D. B. and Suzuki, E. (1973) in Physical Principles and Techniques of Protein Chemistry, Part C (Leach S. J., ed.), vol. 21, pp. 301–355, Academic Press, New York
17 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
18 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
19 Martínez, J., Campetella, O., Frasch, A. C. C. and Cazzulo, J. J. (1991) Infect. Immunol. **59**, 4275–4277
20 Campetella, O., Martinez, J. and Cazzulo, J. J. (1990) FEMS Microbiol. Lett., **67**, 145–150
21 Renlund, S., Klintrot, I.-M., Nunn, M., Schrimsher, J. L., Wernstedt, C. and Hellman, U. (1990) J. Chromatogr. **512**, 325–335
22 Sambrook, J., Fritsch, E. T. and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
23 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
24 Ibáñez, C., Affranchino, J. L. and Frasch, A. C. C. (1987) Mol. Biochem. Parasitol. **25**, 175–184
25 Henriksson, J., Åslund, L., Macina, R. A., Franke de Cazzulo, B. M., Cazzulo, J. J., Frasch, A. C. C. and Pettersson, U. (1990) Mol. Biochem. Parasitol. **42**, 213–224
26 Henriksson, J., Porcel, B., Ridåker, M., Ruiz, A., Sabaj, V., Galanti, N., Cazzulo, J. J., Frasch, A. C. C. and Pettersson, U. (1995) Mol. Biochem. Parasitol. **73**, 63–74
27 McCarthy-Burke, C., Taylor, Z. A. and Busch, G. A. (1989) Gene **82**, 177–189
28 Sanger, F., Niklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
29 Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Methods Enzymol. **185**, 60–89
30 Campetella, O., Henriksson, J., Åslund, L., Frasch, A. C. C., Pettersson, U. and Cazzulo, J. J. (1992) Mol. Biochem. Parasitol. **50**, 225–234
31 Bontempi, E. J., Búa, J., Åslund, L., Porcel, B., Segura, E. L., Henriksson, J., Orn, A., Pettersson, U. and Ruiz, A. M. (1993) Mol. Biochem. Parasitol. **59**, 253–262
32 Valle, F., Becerril, B. L., Chen, E., Seeburg, P. H., Heyneker, H. and Bolivar, F. (1984) Gene **27**, 193–199
33 Bansal, A., Dayton, M. A., Zalkin, H. and Colman, R. F. (1989) J. Biol. Chem. **264**, 9827–9835
34 Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, A. R., Kerlavage, E. F., et al. (1995) Science **269**, 496–512
35 Gillin, F. D., Reiner, D. S. and McCaffery, J. M. (1996) Annu. Rev. Microbiol. **50**, 679–705
36 Cock, J. M., Kim, K. D., Miller, P. W., Hutson, R. G. and Schmidt, R. R. (1991) Plant Mol. Biol. **17**, 1023–1044
37 Kinnaird, J. H. and Fincham, J. R. S. (1983) Gene **26**, 253–260
38 Nagasu, T. and Hall, B. D. (1985) Gene **37**, 247–253
39 Benachenhou, N. and Baldacci, G. (1991) Mol. Gen. Genet. **230**, 345–352
40 Benachenhou-Lahfa, N., Labedan, B. and Forterre, P. (1994) Gene **140**, 17–24
41 Zelada, C., Montemartini, M., Cazzulo, J. J. and Nowicki, C. (1996) Mol. Biochem Parasitol. **79**, 225–228
42 Tomas, A. M. and Kelly, J. M. (1996) Mol. Biochem. Parasitol. **76**, 91–103
43 Bormann, E. R., Eikmanns, B. J. and Sahm, H. (1992) Mol. Microbiol. **6**, 317–326
44 De Zoysa, P. A., Connerton, I. F., Watson, D. C. and Johnston, J. R. (1991) Curr. Genet. **20**, 219–224
45 Hawkins, A. R., Gurr, S. J., Montague, P. and Kinghorn, J. R. (1989) Mol. Gen. Genet. **218**, 105–111
46 Schaap, P. J., Muller, Y., Baars, J. J., Op den Camp, H. J., Sonnenberg, A. S., van Griensven, L. J. and Visser, J. (1996) Mol. Gen. Genet. **250**, 339–347
47 Chavez, S., Reyes, J. C., Chauvat, F., Florencio, F. J. and Candau, P. (1995) Plant Mol. Biol. **28**, 173–188
48 Hein, J. J. (1990) Methods Enzymol. **183**, 626–645

Received 10 July 1997/10 November 1997; accepted 19 November 1997