The gene family of EF-hand calcium-binding proteins from the flagellum of *Trypanosoma brucei*

Yimin WU,*§¶ James DEFORD,†¶ Robert BENJAMIN,† Mary Gou-Shu LEE‡ and Larry RUBEN*¶

*Department of Biological Sciences, Southern Methodist University, Dallas, TX 75235; †Department of Biology, University of North Texas, Denton, TX 76021, and

‡Division of Tropical Medicine, School of Public Health, Columbia University, New York, NY 10032, U.S.A.

The flagellum of *Trypanosoma brucei* contains calmodulin, and a separate family of antigenically related EF-hand calcium-binding proteins which we call calflagins. The following study evaluates the structure and genomic organization of the calflagin family. Genomic Southern blots indicated that multiple copies of calflagin genes occurred in *T. brucei*, and that all of these copies were contained in a single 23 kb *XhoI*–*XhoI* fragment on chromosomes 15 and 16. mRNAs of 1.2 and 1.6 kb were identified in bloodstream and procyclic life-cycle stages. Genomic fragments of 2.5 and 1.7 kb were cloned that encoded calflagin sequences. The calflagin genes were arranged tandemly along the genomic fragments. Three new members of the calflagin family were sequenced from a cDNA clone and the two genomic clones. Two unrelated families of 3' flanking sequences were downstream from the calflagin genes. An open reading frame that was

unrelated to any calflagin sequence was at the 5' end of the 2.5 kb genomic fragment. The deduced amino acid sequences of the genomic clones (called Tb-24 and Tb-1.7g) were similar to the previously described Tb-17. Each encoded an approximately 24 kDa protein which contained three EF-hand calcium-binding motifs and one degenerate EF-hand motif. The cDNA encoded a protein (called Tb-44A) which was approximately twice as large as the other calflagins. The large size resulted from a nearly direct repeat of 186 amino acids. In general, variability among the *T. brucei* calflagins was greater than observed for related proteins from *Trypanosoma cruzi*. We demonstrate that this variability resulted from amino acid substitutions at the N-terminus, C-terminal extensions, and duplication of internal segments.

INTRODUCTION

Trypanosomes of the brucei group are flagellated protozoa that produce lethal infections in humans and livestock throughout sub-Saharan Africa. The complex trypanosome life cycle, which involves stages in an insect and vertebrate host, suggests that trypanosomes have the ability to recognize and respond to signals in their environment. The following study is predicated on the assumption that African trypanosomes utilize calcium signals to co-ordinate life-cycle events during infection. The extent to which this assumption is correct depends upon the variety of proteins within the cell that can respond to calcium signals (reviewed in [1]). In this regard it is of interest that calcium-binding proteins which may modulate calcium signals have been found in the flagellum of *Trypanosoma brucei*.

The trypanosome flagellum is a complex structure that possesses an axoneme, paraxial rod [2,3], and desmosome-like adhesions to the cell surface [4,5]. The flagellum is used as the site of cell motility, cell recognition/adhesion reactions [6], and endocytosis [7–9]. A family of adenylate cyclases has been localized along the flagellum [10]. Activity of the cyclases varies during the trypanosome life cycle [11]. Calcium ions appear to regulate one of the adenylate cyclase isoforms [12]. However, the specific protein that might sensitize the cyclase to calcium remains unclear. In mammalian cells, a calmodulin-dependent adenylate cyclase has been described [13], but no evidence is available to suggest that calmodulin modulates the trypanosome cyclase.

Nonetheless, immunofluorescence has been used to identify calmodulin as a component of the trypanosome flagellum [14], and immunogold labelling has localized calmodulin to the paraxial rod (L. Ruben, P. Webster and C. L. Patton, unpublished work). Spectrin is a calmodulin-binding protein in mammalian cells, and a protein related to spectrin was also identified in the trypanosome flagellum [15,16].

Non-calmodulin calcium-binding proteins have been detected in the trypanosome flagellum. In *Trypanosoma cruzi*, genes for a 24 kDa EF-hand calcium-binding protein have been cloned independently by several groups. The genes have been called 1F8 [17], FCaBP [18], and the 24 kDa antigen [19]. When compared with each other, the deduced amino acid sequences for 1F8 and FCaBP gene products were identical and differed from the 24 kDa antigen in only four amino acids. A new sequence from *T. cruzi* strain PBOL has recently appeared in the database (GenBank accession number L26971) which exhibits six amino acid differences with FCaBP (L. M. Barthel, R. S. Tibbetts, C. L. Olson, B. M. Chaudoir, S. D. Miller and D. M. Engman, unpublished work). Antibodies raised against FCaBP demonstrated that this protein was localized to the flagellum [18], while a ⁴⁵Ca gel-overlay assay demonstrated that FCaBP bound to calcium [18]. Overall, these genes are of interest because they appear to encode a major antigen recognized by Chagasic serum [18,19], and when expressed in bacteria, cause the bacteria to clump [20].

In *Trypanosoma lewisi*, sequences related to the *T. cruzi* flagellar calcium-binding proteins were identified by screening

Abbreviations used: IPTG, isopropylthiogalactoside; ORF, open reading frame; SSC, 0.15 M NaCl/0.015 M sodium citrate; TAFE, transverse alternating-field electrophoresis.

§ Present address: Laboratory of Malaria Research, National Institutes of Health, Bethesda, MD 20892, U.S.A.

¶ To whom correspondence should be addressed.

¶ These authors contributed equally to this study.

expression libraries with serum from infected rats [21]. The *T. lewisi* clone was then used to screen a *T. brucei* cDNA library and the full-length cDNA of Tb-17 was obtained [21]. Tb-17 encoded a 23 kDa protein that contained EF-hand calcium-binding motifs. Tb-17 shared 56% sequence identity with FCaBP and was presumed to reside in the flagellum. Subsequently, a series of proteins that interacted with phenyl-Sepharose in a calcium-dependent way were partially purified from *T. brucei* [22]. These proteins were collectively named trypanosome calcimedins. Some of the calcimedins were antigenically related and consisted of a 44 kDa protein and a cluster of proteins with masses of 23–26 kDa. The 44 kDa calcimedin (called Tb-44) was hydrolysed to produce CNBr/tryptic fragments. Seven of these fragments, representing 18% of the total protein, were sequenced. Each fragment exhibited a high degree of identity with sequences from Tb-17. Immunolocalization and immunoblot analyses identified Tb-44 and the 23–26 kDa cluster in flagella-enriched preparations [22]. Surprisingly, immunoblot analysis demonstrated that only the 24 kDa EF-hand protein was present in *T. cruzi*, and no immunological evidence was obtained for the diversity of protein masses observed in *T. brucei* homogenates [18,22].

In the present report, we examine the molecular basis for the structural differences among the non-calmodulin, flagellar EF-hand calcium-binding proteins. These proteins are shown to include: Tb-17, Tb-24, Tb-44 and Tb-1.7g from *T. brucei*, and 1F8, FCaBP, 24 kDa antigen and 25 kDa FCaBP from *T. cruzi*. As a convenient means of identification, this family of related proteins is herein referred to as the calflagins. The present report describes the sequences, transcription and genomic organization of the *T. brucei* calflagins. The DNA sequences which we present in this report have been submitted to GenBank, and have the following accession numbers: Tb-44A, U06463; Tb-24, U06644; and Tb-1.7g, U05882.

MATERIALS AND METHODS

Proteins

Crude calcimedins were purified from *T. brucei brucei* antigenic type M110 as described previously [22]. Protein separation by SDS/PAGE, immunoblot analyses and antibody production were as described [22].

Labelling of cDNA probes

Radioactive cDNA probes were prepared with the Prime-a-Gene labelling kit (Promega) following the manufacturer's instructions. Non-radioactive biotinylated cDNA probes were prepared with the Stratagene Flash/Prime-It Random Primer Labelling kit following the manufacturer's instructions. The unincorporated nucleotides were removed with a Sephadex G-50 spin column following standard procedures [23]. The purified probes were used directly in hybridization reactions or stored at -20°C for later use.

Screening the λ -Zap library

A λ -Zap cDNA library of bloodstream form *T. brucei* clone IsTAR1 [24] was kindly provided by Dr. K. Stuart, Seattle Biomedical Research Institute, Seattle, WA, U.S.A. The library was screened with the cDNA for Tb-17 following standard Stratagene protocols for the λ -Zap vector. The complete Tb-17 cDNA with *Sal*I ends had been cloned into the *Sal*I site of pUC19 and the entire *Sal*I–*Sal*I fragment of Tb-17 was used as a probe. Fourteen positive clones were identified among 70 000

plaques plated for initial screening and nine positive clones were confirmed by tertiary screening. To select clones that specifically encoded Tb-44, the positive clones were converted into phagemids and the fusion proteins were induced with isopropylthio-galactoside (IPTG), following the manufacturer's instructions (Stratagene). Bacterial lysates containing fusion proteins from these clones were analysed by immunoblot procedures with anti-Tb44 serum. The cDNA insert of plasmid pTB44A was studied further (GenBank accession number U06463).

Genomic clones

Genomic DNA from trypanosome strain 427-60 was digested with *Bam*HI and hybridized with the *Sal*I–*Sal*I complete sequence of Tb-17 cDNA. Fragments of approximate size 1.7 kb and 2.5 kb hybridized to the probe. *Bam*HI-digested genomic DNA of approximate size 1.7 kb and 2.5 kb was excised from agarose gels and subcloned into the *Bam*HI site of pUC19 to produce two size-fractionated libraries. The size-fractionated libraries were screened with the *Sal*I–*Sal*I fragment of cloned Tb-17 cDNA. The genomic clones that were obtained from the size-fractionated libraries were designated pB1.7 (GenBank accession number U05882) and pB2.5 (GenBank accession number U06644).

Isolation of phage and plasmid DNA, subcloning, transformation and DNA sequencing

The complete sequence on both strands was obtained for the cDNA insert of plasmid pTB44A, and genomic DNA inserts of plasmids pB1.7 and pB2.5. The sequencing strategies involved: (a) the production of overlapping fragments with restriction endonucleases; (b) the use of Erase-A-Base (Promega) to shorten the insert DNA; and (c) the synthesis of oligonucleotide primers to fill in gap regions. The Sequenase Version 2 kit (US Biochemical) was used for the sequencing reactions.

Northern-blot analyses

Total RNA was isolated from bloodstream and cultured procyclic forms as described previously [25]. Northern blots were hybridized with ^{32}P probes and washed to a final stringency of $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$: 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS at 65°C .

Genomic DNA and chromosomes

Genomic DNA of size 20–40 kb was isolated as described previously [14]. To obtain chromosome size DNA, cells were harvested from rat blood and suspended in prewarmed (42°C) PBS (57 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , 43 mM NaCl, pH 8.0) to a density of 1×10^8 cells/ml. One volume of prewarmed 1% (w/v) low-melting-point agarose made in PBS containing 50 mM EDTA was added to the cell suspension immediately. The mixed suspension was pipetted into the precooled (4°C) chamber of a gel-casting stand and set at 4°C for 60 min. The agarose–cell blocks were gently pushed out from the gel-casting stand and incubated in lysis buffer (500 mM EDTA, pH 9.5, 1% SDS, containing 2 mg/ml proteinase K) at 50°C overnight with gentle rocking. The blocks were then washed three times with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.5) followed by incubation with lysis buffer containing 1 mg/ml proteinase K at 50°C overnight. Finally the blocks were washed with TE buffer as before and stored in 50 mM EDTA, pH 9.5, at 4°C . The transverse alternating-field electrophoresis (TAFE) system (Beckman) was used to separate chromosomes of *T. brucei*.

<u>AGTTCTGTA</u> <u>CTATATTT</u> <u>TAATCAAGCAAGCCAAGTAAATAACAACG</u>	ATG GGT TGC TCT GCA TCG AAG GAT ACA ACG AAC TCC	87
	M G C S A S K D T T N S	
AAG GAT GGT GCC GCC AGT AAA GGT GGA AAG GAC GGG	AAA ACT ACT GCG GAC CGG AAG GTT GCG TGG GAA CGT ATC	162
K D G A A S K G G K D G	K T T A D R K V A W E R I	
CGG TGT GCT ATT CCC CGT GAC AAG GAT GCT GAG TCG AAA AGC CGT	CGC ATT GAA CTC TTC AAG CCG TTT GAC ACG	237
R C A I P R D K D A E S K S R R I E L F K R F D T		
AAT GGG ACT GGA AAA CTT TCA TTC AGG GAG GTG CTT GAT GGT TGC TAC AGT ATA CTC	AAG TTG GAT GAG TTC ACG	312
N G T G K L S F R E V L D G C Y S I L K L D E F T		
ACT CAC CTT CCT GAC ATT GTC CAG CGT GCG TTT GAC AAA GCG AAG GAC CTC GGC AAC AAG GTA AAG GGC GTG GGT		387
T H L P D I V Q R A F D K A K D L G N K V K G V G		
GAA GAG GAT CTT GTG GAG TTC CTT GAA TTC CGC CTG ATG CTT TGC TAC ATC TAT GAT ATC TTT GAG TTA ACT GTA		462
E E D L V E F L E F R L M L C Y I Y D I F E L T V		
ATG TTC GAC ACC ATG GAT AAG GAT GGA AGT TTG TTG CTT GAG CTA CAG GAG TTC AAG GAA GCC TTG CCG AAA TTG		537
M F D T M D K D G S L L L E L Q E F K E A L P K L		
AAG GAG TGG GGT GTT GAT ATC ACT GAC GCT ACC ACT GTG TTT AAT GAG ATC GAC ACT AAT GGA TCT GGT GTT GTG		612
K E W G V D I T D A T T V F N E I D T N G S G V V		
ACA TTC GAT GAA TTC TCA TGC TGG GCT GTC ACT AAA AAG TTG CAG GTA TGT GGC GAT CCC GAT GGT GAG	GCT GCT	687
T F D E F S C W A V T K K L Q V C G D P D G E	G A	
GCT	AAA ACT ACT GCG GAC CGG AAG GTT GCG TGG GAG CGT ATT CGG TGT GCG ATT CCC CGT GAC AAG GAT GCT GAG	762
A	K T T A D R K V A W E R I R C A I P R D K D A G	
TCG AAA AGC CGT CGT ATT GAA CTC TTC AAG CAG TTT GAC ACG AAC GGG ACT GGA AAA CTC GCG TTC AGG GAG GTG		837
S K S R R I E L F K Q F D T N G T G K L G F R E V		
CTT GAT GGT TGC TAC AGT ATA CTC AAG TTG GAT GAG TTC ACG ACT CAC CTT CCT GAC ATT GTC CAG CGT GCG TTT		912
J D G C Y S I L K L D E F T T H L P D I V Q R A F		
GAC AAA GCG AAG GAC CTC GGC AAC AAG GTA AAG GGC GTG GGT GAA GAG GAT CTT GTG GAG TTC CTT GAA TTC CGC		987
D K A K D L G N K V K G V G E E D L V E F L E E F R		
CTG ATG CTT TGC TAC ATC TAT GAT ATC TTT GAG TTA ACT GTA ATG TTC GAC ACC ATG GAT AAG GAT GGA ACT TTG		1062
L M L C Y I Y D I F E L T V M F D T M D K D G S L		
TTG CTT GAG CTA CAG GAG TTC AAG GAA GCC TTG AAG AAA TTG AAG GAG TGG GGT GTT GAT ATC ACT GAC GCT ACC		1137
L L E L Q E F Q E A L K K L K E W G V D I T D A T		
ACT GTG TTT AAT GAG ATC GAC ACT AAT GGA TCT GGT GTT GTG ACA TTC GAT GAA TTC TCA TGC TGG GCT GTC ACT		1212
T V F N E I D T N G S G V V T F D E F S C W A V T		
AAA AAG TTG CAG GTA TGT GGC GAT CCT GAT GGT GAG	GAG AAC GGA GCA AAT GAA GGT AAT TGA AAGCAGAAGGCATTA	1290
K K L Q V C G D P D G E	E N G A N E G N *	
CTGATGGGTCTGCTTTATGTAATAGAAACGATGCCAAGCGGGCTGTGGGGTCAATGCCCTTAGCCTCGCTTCCTTGATCATATATATCTTGATCATA		1389
TATTCCTTAATTTTTGTCCTGTGTGACCTGTGTGTTTCTCTTTTAAATGTTTGTCTTCCTGTGTTTTCATAACTATCTTAGCCACTGTCCG		1488
CTACAGAAAAAAAAAAAA		1507

Figure 1 Complete nucleotide sequence of clone 1.1B

The 1.5 kb insert of plasmid pTB44A was sequenced to completion. The numbers in the right-hand margin refer to the position of the nucleotides. An ORF encoding a 407 residue polypeptide was identified. Underlined nucleotides indicate part of the mini-exon sequence. Boxed regions are internal direct, imperfect repeat segments. Bold letters in both nucleotide and amino acid sequences indicate the substitutions within the repeats. The single-letter code for amino acids is shown.

Agarose blocks containing trypanosome chromosomes were loaded into the wells of a 0.6% agarose gel prepared with TAFE buffer [10 mM Tris base, 5 mM EDTA (free acid form), 4 mM acetic acid, no adjustment to the pH], and wells were sealed with 0.6% agarose. Electrophoresis was performed at 40 V, 45 min per pulse for 3 h followed by 60 V, 20 min per pulse for 60 h at 12 °C. Chromosomal DNA was visualized with ethidium bromide staining. For electrophoretic transfer of chromosomal DNA on to nylon membranes, the DNA in the gel was first depurinated with 0.2 M HCl for 5 min, followed by denaturation with 0.4 M NaOH for 60 min. After neutralization with 1.5 M NaCl/1 M Tris, pH 7.0, the gel was equilibrated with DNA-blotting buffer (1.25 mM glycine, 1.14 mM NaOH) for 60 min. The electro-

phoretic transfer was conducted with DNA-blotting buffer at 100 V for 4 h at 12 °C.

Hybridization conditions for the genomic Southern blots

Hybridizations were performed in 50% formamide, containing 1 M NaCl, 1% SDS, 10% (w/v) dextran sulphate and 0.5 mg/ml salmon sperm DNA at 42 °C following standard protocols [16]. For the high-stringency wash, 0.1 × SSC was used at 60 °C for 60 min. The membrane was then exposed to X-ray film for 4–12 h. For the repeated hybridization of different probes to the same DNA blot, the membrane was kept wet during auto-

radiography and was stripped afterward with three changes of 0.1 M NaOH, followed by neutralization with 0.1 M Tris, pH 8.0. The stripped membrane was then stored in $2 \times$ SSC until hybridized with the next probe.

RESULTS

Molecular cloning of Tb-44

Immunoblot analysis of trypanosome homogenates identified a structurally related family of proteins that we now call calflagins [22]. However, the molecular basis for differences and similarities among these family members was not known. The 44 kDa calflagin (Tb-44) was of special interest since a calflagin of this size was not found in *T. cruzi*, and this protein was substantially larger than the other calflagins that had been identified in *T. brucei* by immunoblot procedures. Sequences that accounted for this larger size were sought. The complete *SalI-SalI* cDNA clone for Tb-17 was used to screen a λ Zap cDNA library of bloodstream forms of *T. brucei*. To distinguish clones coding for

Tb-44 from clones coding other calflagin family members, the nine positive clones obtained from the tertiary screening were converted into pBluescript phagemids and the recombinant proteins were analysed on immunoblots using antiserum against gel-purified authentic Tb-44 [22]. A 47 kDa protein was expressed by pTB44A. The protein was only present following addition of IPTG, and cross-reacted with antiserum against Tb-44 (results not shown). The complete sequence of the 1.5 kb cDNA insert of pTB44A is shown in Figure 1. Part of the mini-exon sequence [26], which is added by trans-splicing to all mature mRNAs in *T. brucei* was identified at the 5' end of the gene (underlined in Figure 1). The presence of the mini-exon indicates that the cDNA insert of pTB44A contains a complete coding sequence. An open reading frame of 1221 nucleotides was identified. A long stretch of 559 bp within the coding region was imperfectly repeated (boxed residues, Figure 1). Only 12 nucleotide substitutions distinguished the two repeat regions. The repeats were separated by a nine nucleotide hinge sequence which did not appear elsewhere in the gene. The complete open reading frame encoded a putative polypeptide of 407 amino acids. The deduced

GGATCCTTCT	CCGGTTGTTT	ATTAACCTGG	TTATTGTTAG	TATTGAACCT	GCTGTTGGCC	CGAAGCGGT	GCACGCTGTC	GTGAGCCTGA	TTGGTGCTTT	100
ACCGGCAGCT	GTCATGGTTT	TTATTCTACC	AGCGTTGCTC	ACTATGCAGG	TTGACCATGC	TGTAATGAAT	CCCGAAGAGG	ATCGTGTGAC	GTTGAAGTAC	200
TGGGGAAGCA	TGTTTACGGC	AGCACCAGTG	TTTTCTTGGA	CACGCATTGC	TTGTTACTTC	TACATTATCT	TTGGTTTAAT	TGTCATGGTA	ATGGGCACCT	300
ACAGCGTCAT	TGCTGAACCT	TGAGGCGTGG	TGCCTCCGAG	CTGTGGTGTA	CTGCCATCAC	TGGATTTTAA	AAATTTACGG	TACCGTCGTC	ATGTACGCTT	400
CGTATTCTAT	TGTGCTTTTA	GACATTACAA	CGATTATTC	TTAACACACA	AGCTTACGGT	TGCTGGGGTT	GGCCCGCATG	CGGTACGCTA	GCTGTGAGCA	500
CTTTTATTC	AATACGTAAT	ATCAGCGAGG	CGCGCTGGG	TCGATCTTTT	CCCCCTTTGT	TCGCAGGGAT	TTTTTAATAA	CCACCAAAAC	GTTTATTTTT	600
CTCGTAAGCA	CAAACATATG	ATCATTTAAC	TAGGAACGAA	TTTTCTACGT	GAACAACATA	ATAAATCATG	TGTCGCCCT	CCCCGCTGAT	CTTCATCAT	700
TAGATGTGCT	CACCTCTCG	CATGGAGGCA	TGCACTCTGG	GTCCAAGGAT	GATCCCTTGA	GATTACACTT	CATCAAAATC	ACTATAAAAT	TCTTTTCACT	800
TTTCAATCAC	AAGAGGTCTC	ACCATGTTGC	GTCGTATGTT	CATGCTCTCC	TGCTCGGGG	GGTCTGCTC	AGTGTTTAGA	AAACCAAAAT	CTGTGCTTT	900
GGAGCCATTA	TAGGTCGGCA	TCCACGCCGG	GTCGCAGGTG	TTGAGCTTCT	GCCAGTGGGA	GAATGGCTGC	CCGTGTTGGA	GTATAGGCTG	AGTGCTTTTA	1000
CCTCAACCGG	TTTCCCTCTC	GCTCGTGCTC	ATCTTTGATC	AGACTCCACC	TCACTCCCGG	AGCGGGGTCA	AGATTGTAAC	TCCATCACAC	TCCCTTCGCT	1100
GTCACCTATA	TTGTTTCTCT	CCATCGGTTG	TCATTGACTG	GATAAGTTGT	TGCTTGGTTC	GCCCGTGACG	TTGTTGGGCG	GAGGCGCTTT	CGATACACTT	1200
CTTGCCATCG	CATATGAACC	CGCTCGTTGC	TGTAAGCTGT	GCAGAGCCGT	GTTGGTCACC	GTCTCATCGA	TCCACTGGGA	AATGCAACTA	CGATGAACGA	1300
TCITTTGATT	ATCAGTGAAT	TTCCGAAAGA	CTGTAGCACA	ATGTAGAATC	CGTGTGTATT	TTCCAGCTGC	TGCTTGCGCT	GCCACGCAGC	ATCTTCACTG	1400
CCTATGCTAT	GTTCACGCTG	TTCCATTTTT	GTAGAAGTTA	ATCAAGCAAA	GCCAAGTAAA	ATAAACAAG	ATG GGT TGC TCT GCA TCG AAG GAT			1494
							M G C S A S K D			
ACA ACG AAC TCC AAG GAT GGT GCC GCC AGT AAG GGT GGA AAG GAC GGG AAA ACT ACT GCG GAC CGG AAG GTT GCG TGG GAG										1575
T T N S K D G A A S K G G K D G K T T A D R K V A W E										
CGT ATT CGG TGT GCG ATT CCC CGT GAC AAG GAT GCT GAG TCG AAA AGC CGT CGC ATT GAA CTC TTC AAG CAG TTT GAC ACG										1656
R I R C A I P R D K D A E S K R I E L F K Q F D T										
AAT GGG ACT GGA AAA CTC GGC TTC AGG GAG GTG CTT GAT GGT TGC TAC GGT ATA CTC AAG TTG GAT GAG TTC ACG ACT CAC										1737
N G T G K L G F R E V L D G C Y G I L K L D E F T T H										
CTT CCT GAC ATT GTC CAG CGT GCG TTT GAC AAA GCG AAG GAC CTC GGC AAC AAG GTA AAG GGT GTG GGT GAA GAG GAT CTT										1818
L P D I V Q R A F D K A K D L G N K V K G V G E E D L										
GTG GAG TTC CTT GAA TTC CGC CTG ATG CTT TGC TAC ATC TAT GAT ATC TTT GAG TTG ACT GTA ATG TTC GAC ACC ATG GAT										1899
V E F L E F R L M L C Y I Y D I F E L T V M F D T M D										
AAG GAT GGA AGT TTG CTT GAG TTA CAG GAG TTC AAG GAA GCC TTG CCG AAA TTG AAG GAG TGG GGT GTT GAT ATC ACT										1980
K D G S L L L E L Q E F K E A L P K L K E W G V D I T										
GAC GCT ACC ACT GTG TTT AAT GAG ATC GAC ACT AAT GGA TCT GGT GTT GTG ACA TTC GAT GAA TTC TCA TGC TGG GCT GTC										2061
D A T T V F N E I D T N G S G V V T F D E F S C W A V										
ACT AAA AAG TTG CAG GTA TGT GGC GAT CCT GAT GGT GAG GAG AAC GGA GCA AAT GAA GGT AAT TGA AAG CAGAAGGCAT TACTGATGGG										2150
T K K L Q V C G D P D G E E N G A N E G N *										
TTCTGCTTTA TGTAATAGAA ACCGATGCCA AGCGGGCGTG TGGGGTCAAT GCCTTAGCCT CGCTTCCTTG ATCATATATA TCCTGATCAT ATATTCCCTT										2250
AATTTTTTCG TCCTTGTTGA CCTGTTTGTG TTCCTTTTCT TTTAATGTTT GTCTTCCTGT GTTTTGCGATA ACTATCTCAG CCAGTGTCCG CTACAGATCT										2350
ATATCTCGGA ACACATGTGA ATATGCGTCT GTATTTTCCA GCTGCTGCTT GCGCTGCCAC GCTGCATCTC TGTGCTGCC GTTGATGATCA TGCTGTTCCA										2450
TTTTGTAGAA GTTAATCAAG CAAAGCCAAG TAAAATAAAC AAAG ATG GGT TGC TCA GGA TCC										2512
							M G C S G S			

Figure 2 Complete nucleotide sequence of genomic clone pB2.5

The 2.5 kb *Bam*HI–*Bam*HI genomic fragment was cloned in pUC19 and sequenced to completion. ORFs for Tb-24 and gene A (the first 18 nucleotides of a calflagin coding sequence) are indicated by the single-letter amino acid code. The upstream ORF that encodes a putative non-calflagin protein (residues 1–321) has not been marked.

GGA TCC AAG AAC GCA TCT AAC CCC AAG GAT GGT GCC GCC AGT AAG GGT GGA AAG GAC GGG AAA ACT ACT GCG GAC CGG AAG GTT	84
G S K N A S N P K D G A A S K G G K D G K T T A D R K V	
GCG TGG GAG CGT ATT CGG TGT GCG ATT CCC CGT GAC AAG GAT GCT GAG TCG AAA AGC CGT CGC ATT GAA CTC TTC AAG CAG TTT	168
A W E R I R C A I P R D K D A E S K S R R I E L F K Q F	
GAC ACG AAT GGG ACT GGA AAA CTC GGC TTC AGG GAG GTG CTT GAT GGT TGC TAC GGT ATA CTC AAG TTG GAT GAG TTC ACG ACT	272
D T N G T G K L G F R E V L D G C Y G I L K L D E F T T	
CAC CTT CCT GAC ATT GTC CAG CGT GCG TTT GAC AAA GCG AAG GAC CTC GGC AAC AAG GTA AAG GGT GTG GGT GAA GAG GAT CTT	336
H L P D I V Q R A F D K A K D L G N K V K G V G E E D L	
GTG GAG TTC CTT GAA TTC CGC CTG ATG CTT TGC TAC ATC TAT GAT ATC TTT GAG TTG ACT GTA ATG TTC GAC ACC ATG GAT AAG	420
V E F L E F R L M L C Y I Y D I F E L T V M F D T M D K	
GAT GGA AGT TTG TTG CTT GAG TTA CAG GAG TTC AAG GAA GCC TTG CCG AAA TTG AAG GAG TGG GGT GTT GAT ATC ACC GAC GCT	504
D G S L L L E L Q E F K E A L P K L K E W G V D I T D A	
ACC ACT GTG TTT AAT GAG ATC GAC ACT AAT GGA TCT GGT GTT GTG ACA TTC GAT GAA TTC TCA TGC TGG GCT GTC ACT AAA AAG	588
T T V F N E I D T N G S G V V T F D E F S C W A V T K K	
TTG CAG GTA TGT GGC GAT CCT GAT GAT GAG GAG AAC GGA GCA AAT GAA GGT GAC GGG GCA AAT GCA GGG GAT GGA GTT CCT GCG	672
L Q V C G D P D D E E N G A N E G D G A N A G D G V P A	
GCC GAG GGT TCT GCC TAG TGTGAGAAT GTTATGATTA TGCAGGAACG TCAGTATTTG TGGTCTTACC GTAGAGATCT GTGTTTGCTT CTTTTTTGG	770
A E G S A *	
AGGTGTTTT ATGTTCTTTT TAACGCGCTC CGGTCCATAT TATTTAATAT TATTGTTTCA AGTGTTTTTT AAAACTCAAT GGTGTTTTTT GTCTTCTTTT	870
TCCTTATTTT TAACCTGTCT CTTTTGTCAG GTTATTGGCT ATAATATTTT ATATTGATTT TCATGCTTCT TGATAGCCGA CTGCCTAAGC ACTTGTTTTT	970
GTCTACITTTT GGTTCGTAAG CCTGATTATT TCGTTGTTTA TGAATATTCA CTTTTTTCCA TATGTCGCT CCGGTGAACA TCTTTGGGGA CCAGTAGAGT	1070
GCTGCATCGA TGATAATGTG CCAATATATA TATATATTTA TGTATGTATA AGCACACATT TCGCGCAGTG CGCCATGAAG AATGTAAGGA GAATTTGTGG	1170
GGAAATGTTA TGTAAGTACA TGGTGAAGAG AGGTAGGTAA TTTGTGGGAA TGAAAAGGAA AAGCAGGTG GTGACCGACA CACTCGTGGT TTGTAATCTT	1270
TTTCGTTCTA GCAAAATAGT TTTTATTTT TGTACGTGAT CTCTCATGTC ACCGTTGTG TAAATAGATA TACGCTCTTA TGGGCTGTT AGGGAATTC	1370
TGTATGGCGG AGGCGGAGTG CGCTGTTGCG TGTGATTGAG TGGCGTTACA ACGTGAAATG TTATTATTTT GCTTCGCGTC TGCCATATAC TTCTTTACCT	1470
TCTTTTCTTG TTTACGAAGT TGCTTCTGTG GTGGTGAATA GGAAGCAATG GACACGGAGT TGGTCGCGAT CATTGCTGCA TCCGTTTCCT AATGATCTT	1570
AGGACCGCGT TGCTGCTCTT AGCGGATTGT GCTGACCATG GTCCGTATAG ATATAATAC GCCTGTGCGG GATGCAGCAG TGCTAAGGAT AAAACAACAG	1670
GGTACAGGGA TCC	1683

Figure 3 Complete nucleotide sequence of genomic clone pB1.7

The 1.7 kb *Bam*HI–*Bam*HI genomic fragment was cloned in pUC19 and sequenced to completion. The ORF for the calflagin Tb-1.7g is indicated by the single-letter amino acid code.

amino acid sequence contained a corresponding 186 amino acid repeat which accounted for the large size of Tb-44. Only three amino acid differences were found between the repeated segments. Substitutions in seven other codons did not affect the amino acid sequence. A comparison of the deduced amino acid sequence from pTB44A and the microsequencing data obtained from CNBr/tryptic digests of the authentic Tb-44 protein [22] revealed four amino acids that did not match (see Figure 5). The coding region of pTB44A was therefore named Tb-44A to indicate that it may represent an isoform of the authentic Tb-44.

Genomic clones of calflagin family members

Genomic DNA from *T. brucei* strain 427-60 was digested with *Bam*HI. The complete sequence of Tb-17 cDNA [21] was used as a probe. Genomic DNA fragments of 1.2, 1.7, 2.5 and 4.3 kb hybridized to the Tb-17 cDNA (results not shown). The *Bam*HI genomic fragments of 1.7 kb and 2.5 kb were excised from gels, and the size-fractionated DNA was subcloned into the *Bam*HI site of pUC19. The corresponding size-fractionated genomic libraries were screened with Tb-17 cDNA. The cloned 2.5 kb and 1.7 kb genomic fragments (called pB2.5 and pB1.7 respectively) were sequenced to completion (Figures 2 and 3). A schematic diagram of the relationship between conserved coding and flanking regions of the different calflagin clones is shown in Figure 4. Salient features of the genomic clones include an incomplete open reading frame (ORF) of 321 nucleotides

(residues 1–321; Figures 2 and 4) at the 5' end of pB2.5. The putative translation product of this ORF represents the carboxyl portion of a protein that is unrelated to the calflagins or any other protein in the PIR or Swiss Protein databases (translation not shown). Whether this ORF is actually transcribed has not been determined. Downstream from the ORF is a second ORF that shares nearly identical 5' and 3' non-coding regions with Tb-44A, and encodes a 24 kDa protein with extensive sequence similarity to the carboxyl half of Tb-44A (Figures 2, 4 and 5). This new calflagin family member is called Tb-24. The 3' flanking sequences of Tb-24 are identical with the 3' flanking sequences of Tb-44A (Figure 4). This conserved downstream segment is followed by 131 nucleotides that are repeats of a region found at the 5' end of Tb-24 and includes the putative mini-exon acceptor site (Figure 4). Thirty-six nucleotides downstream from the putative mini-exon acceptor site, the first 18 nucleotides of another calflagin gene were observed (gene A, Figure 4). This calflagin differs from Tb-44A, Tb-17 and Tb-24 in the presence of a *Bam*HI site at the 5' end.

The sequence of the 1.7 kb *Bam*HI genomic clone begins at precisely the same location within the calflagin sequence where the 2.5 kb genomic clone ends (Figures 3 and 4). This is suggestive evidence that the 2.5 kb and 1.7 kb genomic fragments are directly linked. The partial sequence for the new calflagin on the 1.7 kb genomic fragment was called Tb-1.7g (Figures 3–5). The downstream non-coding sequences of Tb-1.7g were related to the downstream sequences of Tb-17 (Figure 4).

Total RNA was isolated from bloodstream and cultured procyclic forms of *T. brucei* strain 427-60. The complete sequence of the

The EF-hand motifs in Tb-44A were compared among themselves and with EF-hand 1 from trypanosome calmodulin (TrypCaM). The canonical EF-hand motif is shown above. The symbols are: n, any hydrophobic amino acid; *, any amino acid; X, Y, Z, -X, -Y, -Z refer to calcium co-ordinating amino acids which have an oxygen in their side chains. These include D, E, S, T, N and Q. The -Y co-ordination site utilizes the α -carbonyl oxygen of the peptide bond, and can therefore be any amino acid. The numbers (#) refer to the EF-hands as shown in Figure 5. Bold letters indicate substitutions between the internally repeated EF-hand sequences. Underlined letters indicate residues that differ from the canonical EF-hand motif.

Total RNA from bloodstream form (lane a) and cultured procyclic forms (lane b) were separated in a 1% formaldehyde agarose gel and transferred to nitrocellulose. The filters were hybridized with the cDNA for Tb-17. Filters were washed to a final stringency of $0.1 \times \text{SSC}$ (pH 6.15) and 0.1% SDS at 65 °C. Molecular-mass standards in kb are indicated at the left.

Genomic DNA from *T. brucei* was digested with *Bam*HI (B) or *Kpn*I (K) as indicated, followed by electrophoresis in a 0.6% agarose gel. Individual panels represent autoradiographs of Southern blots hybridized with 17BgI (a) or 44R-4 (b). The position of molecular-mass standards in kb is shown.

Organization of the calflagin genes within the *T. brucei* genome of strain M110 was further evaluated by genomic Southern blots. A hybridization probe that corresponded to the 3' non-translated flanking region of Tb-17 was prepared from the *Bgl*I-*Sal*I fragment of cloned Tb-17 (see Figure 4 for the location of the

Figure 9 Physical linkage of calflagin genes

Panel A, chromosomal assignment of trypanosome calflagin genes. Trypanosome chromosomes were separated by TAFE. Individual lanes represent: a, ethidium bromide-stained gel; b–e, autoradiographs of Southern blots hybridized with 44R-4 (b), 17Bgl (c), 44R-2 (a separate general probe) (d), and α -tubulin (e) respectively. Bars indicate the position of yeast chromosome markers. Arrows indicate the positions of chromosomes 16 and 15. Panel B, Southern-blot analysis of trypanosome calflagin genes using common probe 44R-4. Genomic DNA from *T. brucei* was digested with: *SmaI* (a); *SsrI* (b); *StuI* (c); *XbaI* (d); or *XhoI* (e). The position of λ *HindIII* size markers is shown at the left.

hybridization probes). This probe was called 17Bgl and was capable of detecting flanking sequences of Tb-17 and Tb-1.7g. A general probe was prepared from the 5' *EcoRI*–*EcoRI* fragment that included part of the repeated coding region of Tb-44A (probe 44R-4; Figure 4). 17Bgl and 44R-4 were then hybridized to genomic DNA from slender bloodstream forms of *T. brucei* clone M110 that had been digested with *Bam*HI or *Kpn*I. Neither enzyme cut within the calflagin-coding sequences. The 17Bgl probe recognized two bands (1.7 kb and 4.3 kb) in genomic DNA digested with *Bam*HI, and one band (5.5 kb) with *Kpn*I (Figure 8a). The sequence of the 1.7 kb *Bam*HI genomic fragment is reported in Figure 3. Since the 1.7 kb *Bam*HI fragment contained Tb-1.7g, but not Tb-17, then Tb-17 must be encoded on the 4.3 kb *Bam*HI fragment (Figure 8a), and linked together on the 5.5 kb *Kpn*I fragment. The common probe, 44R-4 hybridized to five bands when the genomic DNA was digested with *Bam*HI (1.2, 1.7, 2.5, 4.3 and > 20 kb), and two bands when the genomic DNA was digested with *Kpn*I (5.5 and 9.6 kb) (Figure 5b). An identical pattern of *Bam*HI fragments was observed when *T. brucei* strain 427-60 was hybridized with a general probe.

In *T. brucei*, many housekeeping genes exist as multiple copies that are tandemly repeated and co-transcribed. Sequence analysis of genomic DNA demonstrated that some of the trypanosome calflagins were also tandemly linked. To test whether the entire calflagin gene family is physically linked, a panel of restriction enzymes that do not cut within the cDNAs for Tb-44A and Tb-17 was used to digest genomic DNA from *T. brucei* strain M110. *Ssr*I cut within the calflagin locus to produce fragments of 17 and 11 kb that hybridized to the 44R-4 general probe on Southern blots (Figure 9; panel B, lane b). By contrast, *Sma*I, *Stu*I, *Xba*I and *Xho*I each generated one fragment that hybridized to 44R-4 on Southern blots (Figure 9; panel B, lanes a, c–e). These data demonstrate that all of the genes encoding calflagins reside within a single locus of roughly 23 kb (Figure 9; panel B, lane e). Chromosomal assignment of the calflagin locus was sought. Chromosome-size DNA was separated by TAFE, immobilized on nylon membranes and hybridized with probes 17Bgl and 44R-4. As predicted, all three probes hybridized to the same chromosomes (Figure 9, panel A). These chromosomes had previously been designated as 15 and 16 [28]. The tubulin probe

(lane e) is a negative control to demonstrate that chromosomes other than 15 and 16 can be detected by our procedures.

DISCUSSION

We report on the sequences, transcription, and genomic organization of EF-hand proteins from the flagellum of *T. brucei*. During this study, several different trypanosome strains were used to obtain structural data on the calflagin family members. The original partial protein sequence of Tb-44 was obtained from strain M110, while the Tb-44A cDNA was cloned from a library of IsTAR1. Tb-17, Tb-24 and Tb-1.7g were all obtained from strain 427-60. Some of the sequence variability we observed may therefore reflect differences between strains. In particular, the few differences between Tb-44 protein and Tb-44A cDNA sequences may result from strain variability compounded by difficulty in microsequencing of cysteine residues in the CNBr/tryptic peptides. However, several lines of evidence indicate that multiple calflagin family members occur within a single trypanosome. These include: (i) the detection of multiple bands by immunoblot analysis of strain M110 [22]; (ii) the detection of Tb-44-related sequences in both the IsTAR1 cDNA library and M110 cell homogenates; (iii) the detection of identical restriction fragments when genomic DNA of strains M110 or 427-60 were hybridized with coding regions of calflagins (Figure 8, and results not shown); and (iv) the cloning of Tb-17, Tb-24 and Tb-1.7g from the same strain. These data are in contrast with *T. cruzi* where genomic Southern blots identified a 900 bp direct repeat upon digestion with *Bam*HI, *Hind*III, *Pst*I or *Sal*I [17], and only a single band on immunoblots [18,22]. Overall, the data for *T. cruzi* suggest that coding sequences and flanking sequences for the calflagins within a single organism are limited.

The different patterns of 3' flanking sequences for the calflagins may help indicate the evolutionary origins of the calflagin variants. For example, Tb-17 and Tb-1.7g are very similar in their coding sequences (including the same N-terminus and C-terminal extensions), share the same 3' non-coding sequences, and are likely variants of a common progenitor (Figure 4). Tb-24 and Tb-44A also share similar coding sequences (including N- and C-termini that are distinct from Tb-17 and Tb-1.7g), and share the same distinct 3' non-coding region, suggesting that Tb-44A arose from a progenitor to Tb-24. In addition, Tb-44A contained a near perfect internal repeat of 559 bp, which indicated that further heterogeneity in the calflagin family arose from gene duplication of internal sequences. The presence of only 12 nucleotide substitutions between the two halves of Tb-44A indicates that the duplication event occurred relatively recently in the evolution of trypanosomes. This suggestion was strongly supported by the observation that *T. cruzi* contained a 23 kDa calflagin, but not the 44 kDa calflagin [13,15]. These data indicate that the duplication event occurred after the lineage for *T. brucei* and *T. cruzi* diverged. Two different mRNA species of 1.2 and 1.6 kb were identified that encoded the calflagins. The larger mRNA probably results from the transcription products of Tb-44A, Tb-17 and Tb-1.7g. Despite the large size differences between the coding regions for Tb-44A and Tb-17, each cDNA was approximately 1.5 kb. Tb-1.7g has downstream sequences that are related to Tb-17, suggesting the use of a common polyadenylation site and common sized transcript. By contrast, the short 3' non-coding intergenic region of Tb-24 suggests that the smaller 1.2 kb mRNA is encoded by this gene.

The presence of multiple EF-hand calcium-binding proteins in cilia and flagella may be a general phenomenon. The flagellum of *Chlamydomonas* contains calmodulin and a separate EF-hand protein [29–31]. Cilia from *Tetrahymena* contain at least three

other EF-hand proteins besides calmodulin [32], while retinal cells that evolved from flagella contain visinin [33] and recoverin [34]. All of the EF-hand proteins of cilia and flagella are structurally divergent and may have evolved to perform special functions. The function of trypanosome calflagins is under investigation. The flagellar location of these calcium-binding proteins suggests a role in the motility of trypanosomatids. However, the requirement of multiple calcium-binding proteins including calflagins and calmodulin for motility is not known. The situation in *T. cruzi* provides evidence that multiple calflagins, especially the larger Tb-44A, is not an absolute requirement for motility. Both immunoblot analyses [18,22] and molecular cloning [17] demonstrate that the *T. cruzi* flagellum functions as a motile apparatus in the absence of Tb-44A. An alternative possibility is that some members of the calflagin family mediate signal/recognition functions of the trypanosome flagellum. Calcium-sensitive adenylate cyclase [12], host-parasite junctional complexes [6], or the cellular interactions required for genetic exchange [35] are potential targets for calflagin regulation. Gene cloning of the trypanosome calflagins will facilitate these functional analyses.

This work was supported by National Institutes of Health grant AI24627.

REFERENCES

- Ruben, L. and Haghighat, N. (1990) in Calcium as an Intracellular Messenger in Eukaryotic Microbes (O'Day, D. H., ed.), pp. 317–342, American Society for Microbiology Press, Washington, DC
- Farina, M., Attias, M., Souto-Padron, T. and DeSouza, W. (1986) *J. Protozool.* **33**, 552–557
- Schlaeppli, K., Defflorin, J. and Seebeck, T. (1989) *J. Cell Biol.* **109**, 1695–1709
- Smith, D. S., Njogu, A. R., Cayer, M. and Jarlfors, U. (1974) *Tissue Cell.* **6**, 223–241
- Yoshikawa, H., Furuki, J. and Takahashi, Y. (1990) *J. Protozool.* **37**, 27–32
- Tetley, L. and Vickerman, K. (1985) *J. Cell Sci.* **74**, 1–19
- Coppens, I., Opperdoes, F. R., Courtoy, P. J. and Baudhuin, P. (1987) *J. Protozool.* **34**, 465–473
- Coppens, I., Baudhuin, P., Opperdoes, F. R. and Courtoy, P. J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6753–6757
- Webster, P. (1989) *Eur. J. Cell Biol.* **49**, 295–302
- Paindavoine, P., Rolin, S., Van Assel, S., Geuskens, M., Jauniaux, J. C., Dinsart, C., Huet, G. and Pays, E. (1992) *Mol. Cell. Biol.* **12**, 1218–1225
- Rolin, S., Paindavoine, P., Hanocq-Quertier, J., Honocq, F., Claes, Y., Le Ray, D., Overath, P. and Pays, E. (1993) *Mol. Biochem. Parasitol.* **61**, 115–126
- Rolin, S., Halleux, S., Van Sande, J., Dumont, J., Pays, E. and Steinert, M. (1990) *Exp. Parasitol.* **71**, 350–352
- Brostrom, M. A., Brostrom, C. O. and Wolff, D. J. (1978) *Arch. Biochem. Biophys.* **191**, 341–350
- Ruben, L. and Patton, C. L. (1987) *Methods Enzymol.* **139**, 262–276
- Schneider, A., Lutz, H. U., Marugg, R., Gehr, P. and Seebeck, T. (1988) *J. Cell Sci.* **90**, 307–315
- Alcina, A., Hargreaves, A. J., Avila, J., Hesketh, J. E. and Fresno, M. (1988) *Cell Biol. Int. Rep.* **12**, 979–985
- Gonzalez, A., Lerner, T. J., Huecas, M., Sosa-Pineda, B., Nogueira, N. and Lizardi, P. M. (1985) *Nucleic Acids Res.* **13**, 5789–5804
- Engman, D. M., Krause, K. H., Blumin, J. H., Kim, K. S., Kirchhoff, L. V. and Donelson, J. E. (1989) *J. Biol. Chem.* **264**, 18627–18631
- Ouaisi, A., Aguirre, T., Plumas-Marty, B., Piras, M., Schoneck, R., Gras-Masse, H., Taibi, A., Loyens, M., Tartar, A., Capron, A. and Piras, R. (1992) *Biol. Cell* **75**, 11–17
- Lizardi, P., Lerner, T. J., Gonzalez, A. and Nogueira, N. (1985) in *Vaccines 85* (Lerner, R. A., Chanock, R. M. and Brown, F., eds.), pp. 67–70, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Lee, M. G., Chen, J., Ho, A. W. M. and D'Alessandro, P. A. (1990) *Nucleic Acid Res.* **18**, 4252
- Wu, Y., Haghighat, N. G. and Ruben, L. (1992) *Biochem. J.* **287**, 187–193
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbor
- Koslowsky, D. J., Bhat, G. J., Perrollaz, A. L., Feagin, J. E. and Stuart, K. (1990) *Cell* **62**, 901–911
- Lee, M. G.-S., Bihain, B. E., Russel, D. G., Deckelbaum, R. J. and Van der Ploeg, L. H. T. (1990) *Mol. Cell. Biol.* **10**, 4506–4517
- Perry, K. L., Watkins, K. P. and Agabian, N. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8190–8194
- Strynadka, N. C. J. and James, M. N. G. (1989) *Annu. Rev. Biochem.* **58**, 951–998
- Gottesdiener, K., Garcia-Anoveros, J., Lee, M. G.-S. and Van der Ploeg, L. H. T. (1990) *Mol. Cell. Biol.* **10**, 6079–6083
- Gitelman, S. E. and Whitman, G. B. (1980) *J. Cell Biol.* **98**, 764–770
- Huang, B., Mengersen, A. and Lee, V. D. (1988) *J. Cell Biol.* **107**, 133–140
- Taillon, B. E., Adler, S. A., Suhan, J. P. and Jarvik, J. W. (1992) *J. Cell Biol.* **119**, 1613–1624
- Takemasa, T., Takagi, T., Kobayashi, T., Konishi, K. and Watanabe, Y. (1990) *J. Biol. Chem.* **265**, 2514–2517
- Yamagata, K., Goto, K., Kuo, C. H., Kondo, H. and Miki, N. (1990) *Neuron* **2**, 469–476
- Dizhoor, A. M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philipov, P. P., Hurley, J. B. and Stryer, L. (1991) *Science* **251**, 915–918
- Tait, A. and Turner, C. M. R. (1990) *Parasitol. Today* **6**, 70–75