Cloning and sequence analysis of a *Trypanosoma cruzi* α-tubulin cDNA

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A cDNA clone derived from the ***Trypanosoma cruzi*** *α-tubulin gene was isolated and sequenced (Tcαtub; L37345). Tcαtub revealed an 87.79% and an 85.36% identity with the DNA sequence of* ***T. brucei*** *and* ***Leishmania****, respectively. This clone was used to study, by Northern blots, α-tubulin gene expression in epimastigotes, cell-cultured derived trypomastigotes and extracellular amastigotes. α-tubulin MRNA levels were the same in epimastigotes and trypomastigotes, however, there was a drastic decrease in amastigotes. This clone could be useful to elucidate the regulatory mechanisms of α-tubulin gene expression during the differentiation of* ***T. cruzi.***

Key terms: *differential expression, mRNA, secondary structure, sequence homology, tubulin, Trypanosoma cruzi, 3' region, 5' region*

Trypanosoma cruzi is the etiological agent that causes Chagas' disease in humans and affects millions of people in endemic areas of Central and South America (WHO, 1991). The life cycle of the parasite comprises four major stages, epimastigotes, metacyclic trypomastigotes, blood stream trypomastigotes and amastigotes; it includes insect and vertebrate hosts. Throughout its developmental differentiation the parasite undergoes a complex series of morphological and biochemical changes (Contreras *et al,* 1985; Pance & Henriquez, 1993). Several reports have shown that, in *T. cruzi,* tubulin genes are differentially expressed during metacyclogenesis (Rondinelli *et al,* 1986; Ürményi *et al,* 1992; Rodríguez *et al,* 1993). However, the molecular mechanisms that regulate these changes are not clear.

To help in the understanding of tubulin gene expression, we cloned a *T. cruzi* α-tubulin cDNA and used it to analyze, by Northern Blots, the expression of this gene in epimastigotes, trypomastigotes and extracellular amastigotes. A λ ZAP-I cDNA library from *T. cruzi*, Silvio X-10/4 clone (Hoft *et al,* 1989) was kindly donated by Dr J Donelson (University of Iowa) and it was screened with an α-tubulin genomic clone from *Leishmania enriettii* (Wirth & Slater, 1983) kindly donated by Dr S Landfear (The Oregon Health Sciences University). A positive clone, with an insert of 1696 bp, was isolated and designated Tcαtub for *T. cruzi* α-tubulin. For expression studies we

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Abbreviations: aa, amino acid(s); **bp**, base pairs; **cDNA**, DNA complementary to mRNA; **Da**, daltons; **mw**, molecular weight; **nt**, nucleotide; **orf**, open reading frame; **tcαtub**, *Trypanosoma cruzi* α-tubulin.

used the Venezuelan EP strain from *T. cruzi*. Epimastigotes were grown in liver infusion triptose broth (LIT) at 28°C (Camargo, 1964) and were collected 4 and 11 days after inoculation, for logarithmic and stationary epimastigotes, respectively. Trypomastigotes were obtained by infection of Vero cells in culture, and extracellular amastigotes were obtained after extracellular incubation of trypomastigotes (Piras *et al*, 1982). For Northern Blot analysis, total RNA was isolated from parasite pellets by the guanidinium thiocyanate method, separated by agarose/formaldehyde gel electrophoresis and transferred to nitrocellulose membranes (Sambrook *et al*, 1989). The filters were hybridized with the *T. cruzi* α-tubulin cDNA insert labeled with [^{32}P] dCTP, and washed as described by Mahmoudi and Lin (1989).

Figure 1 shows the nucleotide and the deduced aa sequences of the cloned α-tubulin cDNA. The nt sequence was determined twice for both strands in the region from nt 1 to 513 and from nt 916 to 1324. The region from nt 514 to 915 was sequence four times in the 5' to 3' direction only. Similarly, the 3' non-coding region was sequenced four times in the 3' to 5' direction. Computer analysis using the program PC-GENE (Intelegenetic) detected a single open reading frame of 1275 bp, encoding a protein of 425 aa residues (Fig 1), with a predicted mw of 46,980 Da. This mw is lower than the average value of M_r = 54,000 calculated from the migration of tubulin proteins from different organisms (De Souza, 1984). The DNA sequence from Tcαtub was compared with that of *T. brucei rhodesiense* (accession #K02836), *Leishmania donovani* (accession #U09612), and the genomic DNA sequence of the *T. cruzi* tubulin gene from the Dm28c clone (accession #M97965). The sequences were aligned using the clustal method of aligning multiple sequences (MegALign program) available with the DNASTAR package for the Apple Mackintosh computer (DNASTAR, Madison, WI, USA).

Figure 2 shows that there is 87.79%, 85.36% and 99.45% DNA identity of Tcαtub with *T. brucei*, *Leishmania* and the different *T. cruzi* clone, respectively. The differences at the DNA level resulted in only three amino acid changes for *T. brucei* and *T. cruzi* DM28c, and 27 aa changes for *Leishmania* (data not shown). This high percentage of homology at the DNA and aa levels is in agreement with the conserved nature of this molecule (rev. in Cleveland *et al*, 1985). In comparison with the other trypanosomatides, Tcαtub is missing a 26 aa segment near the carboxy-terminal region. This deletion could have occurred during the preparation of the cDNA library and accounts for the lower predicted molecular weight of the ORF cloned. In the coding region, a strong codon bias for G (40%) and C (39.2%) was observed in the third codon position compared to A and T (4.93 and 15.72%, respectively). This represents a codon usage of the GHS type (H = A, C, or T, and S = G or C). In studies analyzing several *T. cruzi* genes, Alonso *et al* (1992) showed that the average codon usage was of the GHG type. The difference in the last base is probably due to the large number of genes analyzed in their study. The putative sites for post-translational modification are conserved, a Lys at position 40 for acetylation, and a carboxy-terminal tyr for tyrosination (in bold in Fig 1). The Tcαtub clone has 46 nt at the 5' end and 375 nt at the 3' end of untranslated sequences which are shown in lower case in Figure 1. These sequences do not have homology with other untranslated sequences found in the GeneBank™. The 5' noncoding region has important features that might favour translational initiation, such as an adenine (marked with an asterisk) positioned 3 nt upstream of the AUG (Yamauchi *et al*, 1991), and a stable (22 kcal/mol) secondary structure (marked in bold) that can be formed 16 nt downstream of the AUG (Kozak, 1990). The 3' and 5' untranslated regions have some pyrimidine-rich sequences (underlined), which are now emerging as important motifs for RNA metabolism in trypanosomas (rev. in Morris *et al*, 1993).

The α-tubulin cDNA clone was used to analyze the expression of this gene *in vitro* grown epimastigotes, trypomastigotes and extracellular amastigotes (Piras *et al*, 1982). The results of the Northern Blot are shown in

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                ccgagcttctcttcaaacaccctcaaataaacaccttcttttcaag ATG CGT GAG GCG
                                                                M   R   E   A
ATT TGC ATT CAC ATC GGT CAG GCC GGT TGC CAG GTT GGC AAT GCG TGC TGG GAG CTG
 I   C   I   H   I   G   Q   A   G   C   Q   V   G   N   A   C   W   E   L
TTC TGT CTG GAG CAT GGT ATC CAG CCC GAC GGT GCG ATG CCG TCC GAC AAG ACG ATT
 F   C   L   E   H   G   I   Q   P   D   G   A   M   P   S   D   K   T   I
GGT GTG GAG GAC GAC GCG TTC AAC ACG TTC TTC TCG GAG ACC GGC GCT GGC AAG CAC
 G   V   E   D   D   A   F   N   T   F   F   S   E   T   G   A   G   K   H
GTG CCG CGC GCG GTG TTC CTC GAC CTG GAG CCG ACG GTG GTG GAC GAG ATC CGC ACG
 V   P   R   A   V   F   L   D   L   E   P   T   V   V   D   E   I   R   T
GGG ACG TAC CGC CAG CTT TTC CAC CCC GAG CAG CTG ATC TCC GGC AAG GAG GAT GCG
 G   T   Y   R   Q   L   F   H   P   E   Q   L   I   S   G   K   E   D   A
GCG AAC AAC TAC GCT CGC GGC CAC TAC ACG ATC GGC AAG GAG ATC GTG GAC CTG TGC
 A   N   N   Y   A   R   G   H   Y   T   I   G   K   E   I   V   D   L   C
CTT GAC CGC ATC CGC AAG CTT GCG GAC AAC TGC ACG GGT CTG CAG GGC TTT CTT GTG
 L   D   R   I   R   K   L   A   D   N   C   T   G   L   Q   G   F   L   V
TAT CAC GCC GTG GGC GGT GGC ACG GGC TCT GGC CTT GGT GCA CTG CTG CTG GAA CGC
 Y   H   A   V   G   G   G   T   G   S   G   L   G   A   L   L   L   E   R
CTC TCT GTG GAC TAC GGA AAG AAG TCC AAA CTG GGC TAC ACA GTG TAC CCA TCC CCA
 L   S   V   D   Y   G   K   K   S   K   L   G   Y   T   V   Y   P   S   P
CAG GTG TCA ACG GCT GTG GTG GAG CCC TAT AAC TCG GTG CTC TCG ACG CAC TCA CTG
 Q   V   S   T   A   V   V   E   P   Y   N   S   V   L   S   T   H   S   L
CTT GAA CAC ACC GAT GTT GCG GCG ATG CTT GAC AAT GAA GCA ATC TAT GAT TTA ACT
 L   E   H   T   D   V   A   A   M   L   D   N   E   A   I   Y   D   L   T
CGT CGT AAC CTC GAT ATT GAG CGT CCA ACG TAC ACA AAT TTG AAC CGC CTC ATC GGC
 R   R   N   L   D   I   E   R   P   T   Y   T   N   L   N   R   L   I   G
CAG GTT GTC TCC GCA CTT ACG GCG TCG CTG CGC TTT GAC GGC GCG CTG AAC GTG GAC
 Q   V   V   S   A   L   T   A   S   L   R   F   D   G   A   L   N   V   D
CTG ACG GAG TTC CAG ACC AAC TTG GTG CCG TAC CCG CGT ATC CAC TTT GTG CTG ACG
 L   T   E   F   Q   T   N   L   V   P   Y   P   R   I   H   F   V   L   T
AGC TAC GCG CCA GTG ATC TCG GCG GAG AAG GCA TAC CAC GAG CAA CTC TCC GTT TCG
 S   Y   A   P   V   I   S   A   E   K   A   Y   H   E   Q   L   S   V   S
GAG ATC TCG AAC GCG GTG TTT GAG CCG GCG TCG ATG ATG ACG AAG TGC GAC CCC CGC
 E   I   S   N   A   V   F   E   P   A   S   M   M   T   K   C   D   P   R
CAC GGC AAG TAC ATG GCA TGC TGC CTC ATG TAC CGC GGC GAC GTT GTG CCG AAG GAC
 H   Q   K   Y   M   A   C   C   L   M   Y   R   G   D   V   V   P   K   D
GTG AAC GCT GCC GTC GCG ACC ATC AAG ACG AAG CGC ACG ATT CAG TTT GTG GAC TGG
 V   N   A   A   V   A   T   I   K   T   K   R   T   I   Q   F   V   D   W
TCG CCC ACT GGC TTC AAG TGC GGC ATC AAC TAC CAG CCC CCT ACG GTG GTG CCC GGT
 S   P   T   G   F   K   C   G   I   N   Y   Q   P   P   T   V   V   P   G
GGC GAC CTT GCC AAG GTG CAG CGC GCG GTG AGC ATG ATT GCC AAC TCG ACC GCC ATC
 G   D   L   A   K   V   Q   R   A   V   S   M   I   A   N   S   T   A   I
GCG GAG GTG TTC GCA CGC ATC GAC CAC AAG TTC GAC CTT ATG TAC AGC AAG CGT GCG
 A   E   V   F   A   R   I   D   H   K   F   D   L   M   Y   S   K   R   A
TTC GTG CAC TGG TAC GTT GGT GAG GGT ATG GAG GAG GGT GAG TTC TCG GAG GCC CGT
 F   V   H   W   Y   V   G   E   G   M   E   E   G   E   F   S   E   A   R
GAG GAG TAC tag agtgggcctcccggcgtctctctctctctttctctttttttttgtttgttcttga
 E   E   Y
ttgattgatttctcttttgtgtgtttccccgcggacgccagcgcggcaaatagggaagaaggaagggacgggcgt
ttaacatttttggggaatgggatgtaggaggggagagaggaggggggaagggacgccgaggagctgcggcaacgtg
ctgggatggtggtttggtggcggtggttgtgtctggcactttcccgccgtgaacgcctcctcgatttccaatgcc
tctaattttccccatttttttttttgattttatttttgtatttgggtcttgtgagtctgttttctccgccccctca
aaaaaggaatttc
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Fig 1. Nucleotide sequence of Tcαtub cDNA and deduced amino acid sequence. Initiation and termination codons are shown in bold. Pyrimidine rich sequences are underlined. Lower case represents non-coding regions. Asterisk indicates an adenine that favour translational initiation and the sequence that can form a stable secondary structure are shown in bold (see text). Lys at position 40 and Tyr at C-terminal end are shown in bold. This sequence has been registered in the GenBank™ under the accession number L37345.

Figure 3A. The gel was stained with ethidium bromide prior to the transfer, to account for loading differences (Fig 3B). Figure 3A shows that the cDNA hybridized with a major band of 2,4 kb and with a minor band of 2.0 kb. Using clone 14 of the CL strain of *T.*

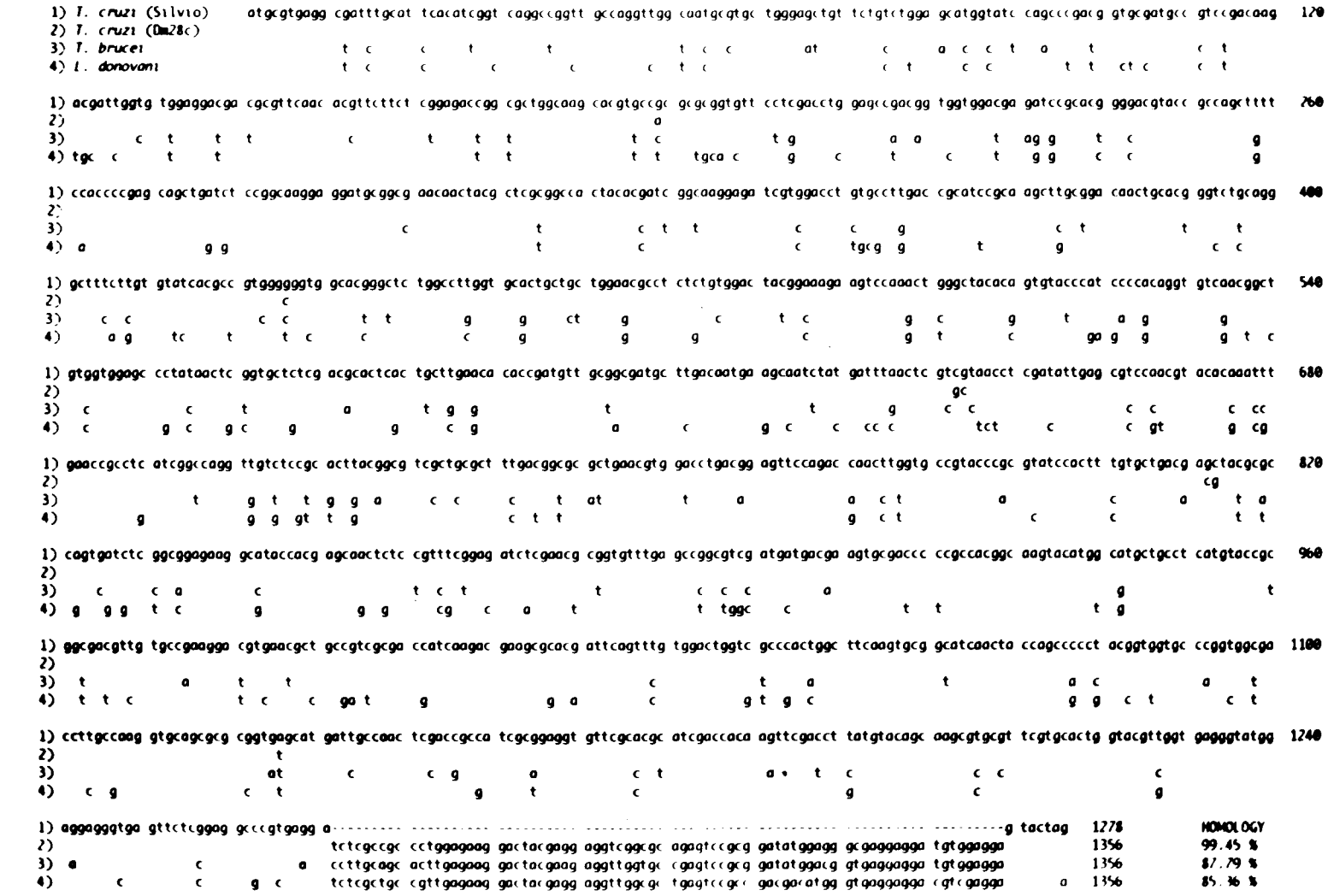

Fig 2. Comparison of nt sequences of α-tubulin. DNA sequences of *T. cruzi* (Silvio), *T. cruzi* (DM28c), *T. bruzei* and *Leishmania* were aligned using the MegAlign program (see text). The blank under each line represents identical nt. Dashes represent arbitrary gaps inserted to maximize homology and one dash corresponds to one nt. The homology was calculated without the missing 78 nt.

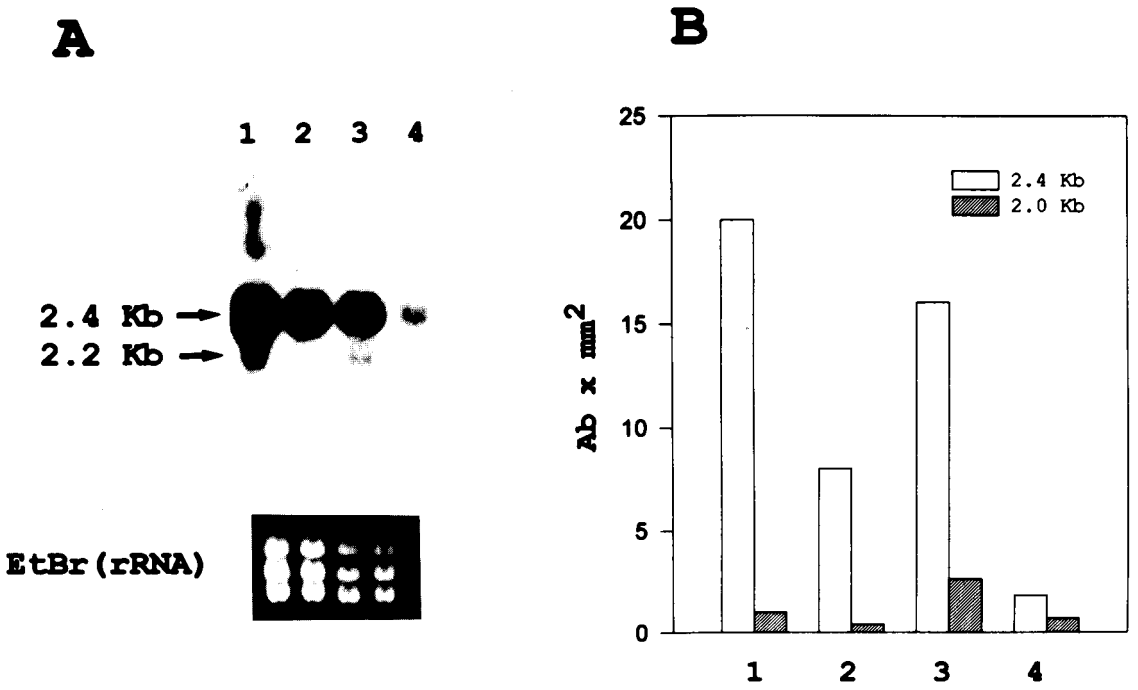

Fig 3. α–tubulin mRNA levels. **A.** Total RNA from logarithmic epimastigotes (1), stationary epimastigotes (2), trypomastigotes (3) and amastigotes (4) was separated on agarose/formaldehyde gels and transferred to nitrocellulose filters. Northern Blots were hybridized with the α–tubulin insert and exposed to X-ray film. **B.** The autoradiogram in A was scanned and the results were normalized with respect to the rRNAs stained with EtBr.

cruzi, Rondinelli *et al* (1986) found one α-tubulin mRNA with a mw of 1.6 kb. Ürményi *et al* (1992) used the same CL strain of *T. cruzi* and reported two mRNA species of 1.8 and 2.2 kb. In the Venezuelan EP isolate used in this work, we found α-tubulin mRNA's of 2.0 and 2.4 kb in agreement with the results of Ürményi *et al* (1992).

The autoradiograms of Figure 3A were scanned, the results corrected with the intensities of the ethidium bromide stained rRNA's, and the results presented in Figure 3B. The figure shows that in comparison logarithmic epimastigotes there is approximately a 2.5-fold decrease in both mRNA species in stationary epimastigotes, while there is a slight decrease in the 2.4 band and a 2.5-fold increase in the 2.0 kb species in trypomastigotes. When trypomastigotes differentiate extracellularly to amastigotes there is an 8-fold and a 4-fold in high and low mw mRNA bands, respectively. The differential expression of high and low mw α-tubulin mRNA's is in agreement with the reports of Ürményi *et al* (1992), that found that during metacyclogenesis there is a 5-fold decrease in high mw RNA, while low mw band decreased only by 50%. A decrease in tubulin mRNA levels during metacyclogenesis has also been reported by Rondinelli *et al* (1986) and Rodriguez (1989). In addition, Ürményi *et al* (1992) found that during metacyclogenesis the changes in α-tubulin gene expression are controlled at the transcriptional and post-transcriptional levels.

Epimastigotes of the EP strain used in this study, do not differentiate *in vitro* to metacyclic trypomastigotes. However, we performed our studies during the growth curve of epimastigotes and observed a drastic decrease in α-tubulin mRNA levels, in the stationary form of the parasite. Ours is the first study of α-tubulin gene expression in tissue-culture derived trypomastigotes and extracellular amastigotes. The decrease observed in extracellular amastigotes is interesting when considering that when trypomastigotes differentiate extracellularly to amastigotes, they alter their morphology to become round and aflagellated (Piras *et al*,

1982). This loss in the flagella could account for the decrease in α-tubulin mRNA levels. We are in the process of determining the molecular mechanisms that regulate the differential expression of α-tubulin by investigating whether the decrease in mRNA levels in stationary epimastigotes and in extracellular amastigotes, is caused by changes in transcriptional rates of the gene, as partly occurs during metacyclogenesis (Ürményi *et al*, 1992). It is possible, however, that the expression of this gene is only controlled post-transcriptionally, as has been suggested for other *T. cruzi* genes like amastin (Teixeira *et al*, 1995). If this were the case, it would be important to determine the role, if any, of the pyrimidine-rich region which we found is present in the 3' untranslated regions present in the gene.

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REFERENCES

ALONSO G, GUEVARA P, RAMÍREZ JL (1992) Trypanosomatidae codon usage GC distribution. Mem Inst Oswaldo Cruz 87: 517-523

CAMARGO EP (1964) Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomas in liquid media. Rev Inst Med Trop São Paulo 6: 93-100

CLEVELAND DW, SULLIVAN KF (1985) Molecular biology and genetics of tubulin. Annu Rev Biochem 54: 331-365

CONTRERAS VT, MOREL CM, GOLDENBERG S (1985) Stage specific gene expression precedes morphological changes during *Trypanosoma cruzi* metacyclogenesis. Mol Biochem Parasitol 14: 83-96

DE SOUZA W (1984) Cell biology of *Trypanosoma cruzi*. Intl Rev Cytol 86: 197-283

HOFT DF, KIM KS, OTSON K, MOSER DR, YOST WJ, BLUMING JH, DONELSON JE, KIRCHOFF LV (1989) *Trypanosoma cruzi* expresses diverse repetitive protein antigens. Infect Immun 57: 1959-1967

KOZAK M (1990) Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. Proc Natl Acad Sci USA 87: 8301-8305

MAHMOUDI M, LIN KV (1989) Comparison of two different hybridization systems in Northern transfer analysis. Biotechniques 7: 331-333

MORRIS DR, KAKEGAWA T, KASPAR RL, WHITE MW (1993) Polypyrimidine tracts and their binding proteins: regulatory sites for posttranscriptional modulation of gene expression. Biochemistry 32: 2931-2937

PANCE A, HENRIQUEZ D (1992) Changes in the proteolytic activity during the growth of *Trypanosoma cruzi* epimastigotes. Biochem Intl 27: 613-623

PIRAS MM, PIRAS R, HENRIQUEZ D (1982) Changes in morphology and infectivity of cell culture-derived trypomastigotes of *Trypanosoma cruzi*. Mol Biochem Parasitol 6: 67-81

RODRIGUEZ F (1989) *Trypanosoma cruzi*: control de la expresión durante la diferenciación celular. Ph.D. Thesis. Universidad Central de Venezuela. Facultad de Ciencias, Escuela de Biología, Departamento de Biología Celular

RODRIGUEZ F, RAMIREZ JL, RANGEL ALDAO R (1993) Differential turn-over of β-tubulin during the cell differentiation of *Trypanosoma cruzi*. Biol Res 26: 35-40

RONDINELLI E, MOURA-NETO RS, SILVA R, SOARES C MA, CARVALHO JF, CASTRO FT (1986) Control of tubulin gene expression during metacyclogenesis of *Trypanosoma cruzi*. FEBS Lett 208: 379-385

SAMBROOK J, FRITSCH EF, MANIATIS T (1989) In: Molecular cloning: A Laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, NY: Cold Spring Harbor Laboratory. pp

SANGER F, NICKLEN S, COULSON AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467

TEIXEIRA SMR, KIRCHHOFF LV, DONELSON JE (1995) Post-transcriptional elements regulating expression of mRNAs from the Amastin/Tuzin gene cluster of *Trypanosoma cruzi*. J Biol Chem 270: 22586-22594

ÜRMÉNYI TP, CASTRO F, CARVALHO JF, DE SOUZA W, RONDINELLI E (1992) Transcriptional and post-transcriptional control of tubulin gene expression in *Trypanosoma cruzi*. DNA Cell Biol 11: 101-109

WORLD HEALTH ORGANIZATION (WHO) (1991) Tropical Diseases: Progress in Research 1989-1990. Geneva, Switzerland: WHO

WIRTH DF, SLATER C (1983) Isolation and characterization of an α-tubulin gene from *Leishmania enriettii*. Mol Biochem Parasitol 9: 83-92

YAMAUCHI K (1991) The sequence flanking translational initiation site in protozoa. Nucleic Acids Res 19: 2715-2720