Control of tubulin gene expression during metacyclogenesis of *Trypanosoma cruzi*

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During differentiation of the dividing epimastigote to the non-dividing metacyclic trypomastigote form of the parasitic protozoan *Trypanosoma cruzi* there is a marked reduction in the rate of synthesis of the major proteins α- and β-tubulin. Our results indicate that the control of synthesis of these proteins during the differentiation event is exerted at the level of α- and β-tubulin mRNA accumulation.

(Trypanosoma cruzi) *Metacyclogenesis* *Tubulin gene* *mRNA*

1. INTRODUCTION

The biological life cycle of the digenetic parasitic protozoan *Trypanosoma cruzi*, the causal agent of Chagas disease, takes place alternatively in an insect and in a mammalian host [1]. Three developmental forms of the parasite appear in the insect: epimastigotes, amastigotes and metacyclic trypomastigotes [1]. The first two forms divide in the gut of the host and give rise to the non-dividing metacycle which is the infective form for the vertebrate. Epimastigotes can be cultivated in liquid acellular media, where they form trypomastigotes [2].

Three papers that appeared in the last two years dealt with the control of tubulin gene expression during form transition in the trypanosomatids of the genus *Leishmania* [3–5]. In these organisms the transformation of amastigotes into promastigotes is accompanied by a marked increase in tubulin biosynthesis. The tubulin gene expression in *L. mexicana* [3,4] was shown to be regulated at the post-transcriptional level by in vitro mRNA translation and Northern blot analysis, using heterologous probes. On the other hand, experiments with *L. enrietii* [5], in which homologous probes were utilized, evidenced that the control of tubulin gene activity is exerted at the level of mRNA accumulation. In the present paper results are reported that show, when homologous genomic cloned DNA of α- and β-tubulin genes are used, that a significantly higher amount of the corresponding tubulin mRNAs is found in epimastigote than in trypomastigote cells of *T. cruzi*. This result is indicative of a control of tubulin gene expression, during metacyclogenesis, operating at the level of tubulin mRNA accumulation. The higher level of tubulin gene expression in the dividing epimastigote cells than in the non-dividing metacyclic trypomastigote cells might be expected considering that α- and β-tubulin are important constituents of the cell division apparatus as well as of the subpellicular microtubules, a structure particularly abundant in trypanosomatids [6].

2. MATERIALS AND METHODS

2.1. *Cultivation and differentiation of* T. cruzi

Clone 14 of the CL strain of *T. cruzi* was used [7]. Organisms of this clone may yield, depending on the cultural conditions (see below), a high pro-

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portion of either epimastigotes or trypomastigotes. The organisms were maintained in LIT medium [2] as epimastigotes, with a small proportion of metacyclic trypomastigotes (5 to 10%), at 29°C. Experimental cultures were started with an inoculum of 2×10^7 organisms/ml in LIT medium. Transfers were performed every 48 h after the population density reached approx. 6×10^7 cells/ml. High rates of differentiation of epimastigotes into metacyclic trypomastigotes were obtained by transferring organisms from a 2-day culture in LIT to M16 medium [7] (0.4% NaCl, 0.04% KCl, 0.8% Na_2HPO_4, 0.2% glucose, 0.125% tryptone from Oxoid, 2.5% inactivated bovine serum, 2% hemoglobin, pH 6.7). By the 5th day, the number of parasites was of the order of 6×10^7 cells/ml and the proportion of trypomastigotes reached 75–90%.

2.2. *Purification of metacyclic trypomastigote by DE-52 resin chromatography*

In order to enrich further a culture with metacyclic trypomastigotes (99%), an aliquot of a culture containing approx. 76% metacyclic trypomastigotes was centrifuged at $5000 \times g$ for 10 min, washed twice in 58.2 mM Na_2HPO_4; 3 mM NaH_2PO_4; 43.5 mM NaCl; 55.5 mM glucose and passed through a column of 6 cm high by 1.5 cm wide of Whatman DE-52 resin previously equilibrated in the same buffer [8]. Metacyclic trypomastigotes were eluted by washing the column with the buffer above. The cells were concentrated by centrifugation as described before and resuspended in M16 medium.

2.3. *Immunoprecipitation of α- and β-tubulin*

The tubulins were identified by immunoprecipitation [9] of in vivo continuously labelled proteins of a 70% metacyclic trypomastigote culture with monoclonal antibodies for α- and β-tubulin (Amersham). Protein A bearing *Staphylococcus aureus* was used as immunoadsorbent.

2.4. *Analysis of in vivo and in vitro labelled proteins of epimastigote and trypomastigote cells*

Cells were continuously or pulse labelled for 17 and 1 h respectively, with 250 μCi/ml of [^{35}S]methionine in their respective culture media. Poly(A)$^+$ polysomal RNAs from epimastigotes and metacyclic trypomastigotes, 0.5 μg each, were translated in an mRNA-dependent translation system from reticulocyte lysate [10]. The radioactive proteins were analysed on a 10% PAGE [11].

2.5. *RNA extraction, isolation and quantitation of poly(A)$^+$ RNA fractions*

Total RNA, polysomal and post-polysomal poly(A)$^+$ RNA, as well as poly(A)$^-$ polysomal RNA were prepared from epimastigote and from 87% trypomastigote cultures. Total RNA was extracted [12] and purified by LiCl precipitation. Post-mitochondrial fraction [13], polysomal and post-polysomal fractions were prepared and the RNAs extracted as described by Shepherd and Nemer [14]. The poly(A)$^+$ RNA fractions were obtained by chromatography in oligo(dT)-cellulose column. The amount of poly(A) containing RNAs was quantitated by hybridization with [^{3}H]poly(U) as described by Rosbach and Ford [15].

2.6. *Nucleic acid electrophoresis, transference to nitrocellulose filters and molecular hybridizations*

RNAs were denatured by the formamide-formaldehyde method and submitted to electrophoresis on 1.2% agarose formaldehyde gels [16]. The RNAs were transferred to nitrocellulose filters as described by Thomas [17]. The ethidium bromide staining of the gel with 1 μg/ml in H_2O was performed in a section cut out of the gel before transference to nitrocellulose filter. Recombinant plasmid DNA pDmT α1 [18] and pβ2 [19] were a gift from Dr Weresink and Dr J.V. Ruderman, respectively. The homologous DNA clone λ Tc3 has been isolated from our genomic library and mapped by Moura-Neto [20]. The λ Tc3 clone contains a 11.5 kb *Eco*RI *T. cruzi* DNA fragment inserted in Charon IV A lambda DNA. The λ Tc3 DNA was prepared as described by Maniatis et al. [16]. Recombinant DNAs were labelled in vitro by nick-translation as described by Rigby et al. [21]. Restriction fragments of λ Tc3 were produced by digestion with the restriction enzymes *Alu*I and *Hae*III according to the manufacturer's instruction and submitted to 1% agarose gel electrophoresis in 50 mM Tris-borate, pH 8.0; 1 mM EDTA and transferred to nitrocellulose filters as described by Southern [22]. The nucleic acids bound to the

nitrocellulose filters were baked for 2 h at 80°C in a vacuum oven and pre-hybridized in a medium consisting of 50% formamide, 100 μg/ml denatured salmon sperm DNA, 5 × SSC, 5 × Denhardt solution and 50 mM Na_2HPO_4 buffer, pH 6.8. High excess of nick-translated DNA was used for RNA blots. The recombinant DNA probes were boiled for 10 min in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA and then added to 15 ml of the above hybridization medium in which 0.1% SDS was included. The heterologous DNA hybridizations were done at 37°C while the homologous DNA hybridization was at 45°C. The filters were washed in 2 × SSC, 0.1% SDS at room temperature for 15 min followed by a 30 min wash in 0.1 × SSC, 0.1% SDS at 37°C for the heterologous hybridization and at 50°C for the homologous hybridizations. The filters were autoradiographed with Kodak X-ray film, using an intensifying screen (lightning plus, Du Pont) at −70°C.

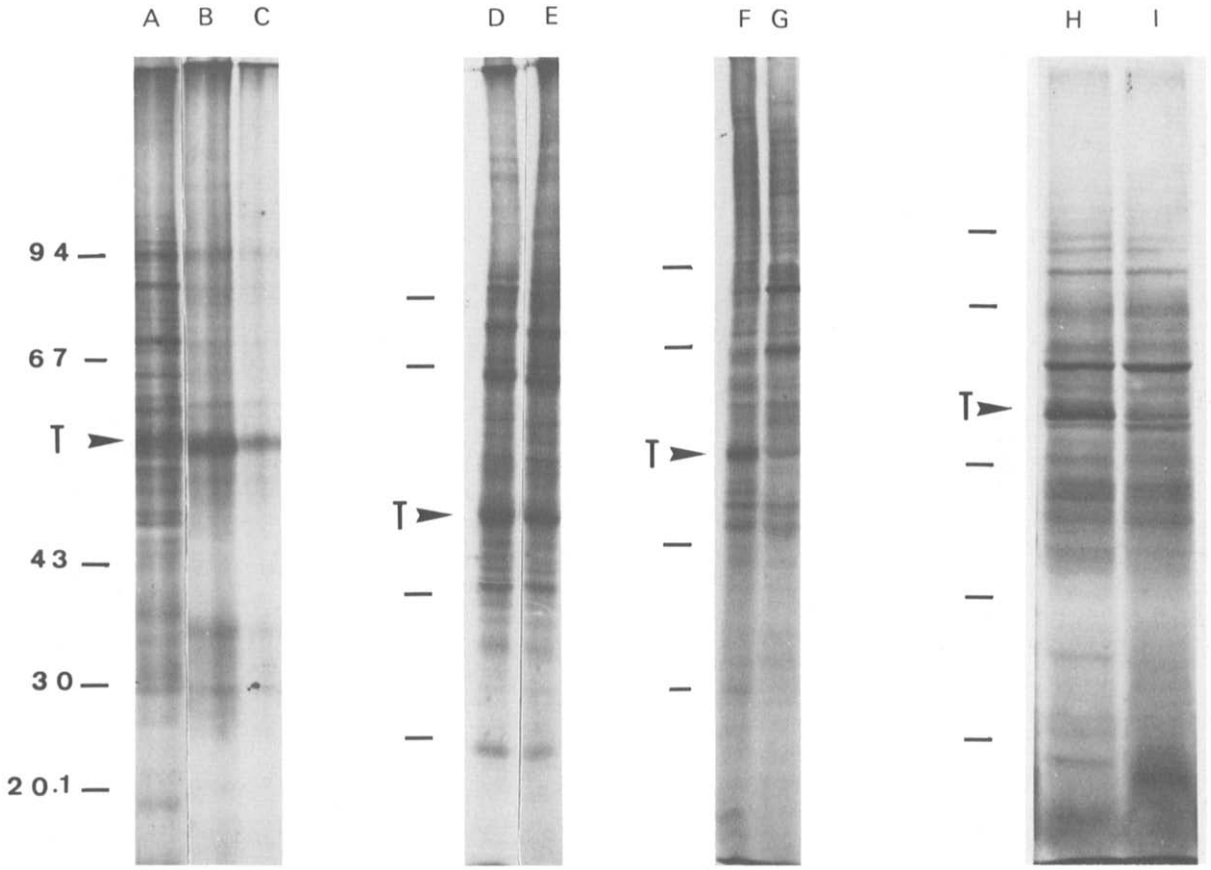

Fig.1. In vivo labelled proteins of epimastigotes and trypomastigotes and in vitro translation products of the respective cell mRNAs. The [^{35}S]methionine labelled proteins (250 μCi/ml) were separated in 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. (A) Total in vivo continuously labelled (17 h) proteins of trypomastigotes; (B) immunoprecipitated β-tubulin; (C) immunoprecipitated α-tubulin; (D) 1 h in vivo pulse labelled proteins of epimastigote and (E) from a culture containing 76% trypomastigote forms; (F) 1 h in vivo pulse labelled proteins of epimastigotes and (G) DE 52 purified trypomastigotes; (H) cell-free products of epimastigotes mRNA and (I) trypomastigote mRNA from an 87% trypomastigote culture. Numbers on the left side indicate the molecular mass protein markers (kDa): phosphorylase *b* (94); bovine serum albumin (67); ovalbumin (43); carbonic anhydrase (30) and soybean trypsin inhibitor (20.1). Arrows indicate tubulin band.

3. RESULTS

3.1. *In vivo and in vitro synthesis of α- and β-tubulin from epimastigote and metacyclic trypomastigote cells*

As shown in fig.1A–C the immunoprecipitated α- and β-tubulins possess very similar molecular masses (~55 kDa) and were not separated in our SDS-PAGE system. Other bands that appear in the gel may result from coprecipitated microtubule associated proteins (MAPs) [23]. The comparison of the rate of synthesis of tubulin in epimastigotes and metacyclic trypomastigotes was made by pulse labelling these cells for 1 h as described in section 2. In fig.1D–G qualitative and quantitative differences can be seen between the pulse labelled proteins of epimastigotes and metacyclic trypomastigotes. In the cultures containing either 75% or 99% metacyclic trypomastigotes, the relative amount of tubulin synthesized was higher in epimastigote than in either of the trypomastigote cultures (fig.1D–G). In order to verify whether this result stemmed from a difference in the level of the specific mRNA available in each parasite form, polysomal poly(A)$^+$ RNA was extracted from epimastigotes and from a culture containing 87% metacyclic trypomastigote forms. Equal amounts of [^{3}H]poly(U) titered poly(A)$^+$ RNA (approx. 0.5 μg) were used to direct the protein synthesis in a rabbit reticulocyte cell-free system. As is readily seen in fig.1H and I, the relative amount of tubulin formed was noticeably higher in the reticulocyte translation products of epimastigote mRNAs than in those of trypomastigote mRNAs. The densitometric ratio of the tubulin bands in the autoradiograms in fig.1F–I is shown in table 1 (rows a,b). The amount of tubulin synthesized was found to be 2-fold higher in epimastigotes than in metacyclic trypomastigotes. These results indicate that, during metacyclogenesis, the amount of tubulin mRNAs was selectively reduced and that this event corresponded to a lower level of translatable tubulin mRNAs. The results described above thus seem to favor the idea that the rate of tubulin synthesis in *T. cruzi*, during metacyclogenesis, reflects the availability of the template.

Table 1

Quantitation of tubulin RNA in epimastigotes and metacyclic trypomastigotes

	Epimastigote/ trypomastigote ratio
a. In vivo translation	2.1:1.0
b. In vitro translation	2.1:1.0
c. Total cell RNA	4.0:1.0
d. Polysomal poly(A)$^+$ RNA	4.8:1.0

Autoradiograms in figs 1F–I and 2C–H were quantitated by densitometry. The ratio of the integrated areas under the peaks was calculated

3.2. *Identification and quantitation of α- and β-tubulin mRNAs in epimastigotes and metacyclic trypomastigotes*

In order to make a direct measurement of the amount of α- and β-tubulin mRNAs in epimastigote and metacyclic trypomastigote cells of *T. cruzi*, total RNA, polysomal and post-polysomal poly(A)$^+$ RNA, were prepared from epimastigote and from an 87% trypomastigote culture. Equal amounts of polysomal and post-polysomal polyadenylated RNAs, titered with [^{3}H]poly(U), were applied to denaturing agarose gels. The RNAs were transferred to nitrocellulose filters and probed with the heterologous tubulin probes: pDm Tα1 [18] (*Drosophila melanogaster*) and pβ2 [19] (*Lytechinus pictus*). The tubulin probe hybridized to one RNA species of 1.6 kb and the β-tubulin probe hybridized to two species of 2.0 and 1.6 kb (fig.2A and B). The relative amount of the tubulin mRNAs was quantitated, during metacyclogenesis, with our homologous genomic DNA clone (λ Tc3), containing sequences encoding both α- and β-tubulin mRNAs (see below). The amount of tubulin mRNAs was appreciably higher in total and polysomal poly(A)$^+$ RNA from epimastigote than in the RNAs from trypomastigote cells (fig.2C–E and G). The amount of rRNA in the total RNA preparations was shown to be comparable by staining two identical aliquots (fig.2J and K) of the same RNA preparations, shown in fig.2C and D, with ethidium bromide. The large rRNA of trypanosomatids is very unstable and dissociates into smaller components [24]. The 26 S *T. cruzi* rRNA gives rise to the two faster migrating components of 2.3 and 1.8 kb shown in fig.2J and K. The upper band corresponds to the 21 S rRNA of 2.8 kb. The

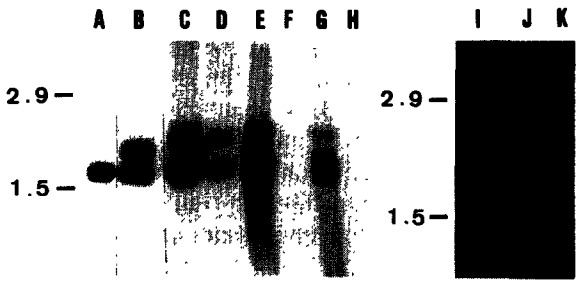

Fig.2. Determination of the relative amount of α- and β-tubulin mRNA in cellular compartments of epimastigotes and trypomastigotes. RNAs were analysed by Northern blotting of 1.2% agarose formamide-formaldehyde gel electrophoresis. 1 μg trypomastigote cytoplasmic poly(A)$^+$ RNA hybridized to (A) pDmTα1 [^{32}P]ATP labelled by nick-translation and (B) pβ2, respectively. (C) 2.5 μg total RNA from epimastigote and (D) from 87% trypomastigote culture hybridized to the nick-translated homologous probe λ Tc3. (E and G) Equal amounts of [^{3}H]poly(U) titered polysomal poly(A)$^+$ RNAs from epimastigote and from an 87% trypomastigote culture, respectively, and (F,H) post-polysomal poly(A)$^+$ RNAs from epimastigote and from an 87% trypomastigote culture, respectively, hybridized to the homologous probe λ Tc3. (I) Ethidium bromide staining of 2.5 μg *E. coli* rRNA, (J,K) ethidium bromide staining of total RNA preparations from epimastigote and metacyclic trypomastigotes used in lanes C and D. Numbers on the left side indicated the size, in kilobases, of *E. coli* rRNA.

equal amounts of rRNA present in epimastigotes and metacyclic trypomastigotes make the difference in amounts of α- and β-tubulin transcripts found between the two forms significative. In table 1 (rows c,d) the densitometric ratio of the α- and β-tubulin mRNA bands in the autoradiograms in fig.2C–H is shown. The amount of α- and β-tubulin mRNA was shown to be 4-fold higher in epimastigotes than in metacyclic trypomastigote cells. Neither epimastigotes nor trypomastigotes presented detectable amounts of tubulin mRNAs in their post-polysomal poly(A)$^+$ RNAs (fig.2F and H). Comparable diminishing amounts of α- and β-tubulin mRNAs were found in the poly(A)$^-$ polysomal RNA fraction of both cell types (not shown). In addition, heavier exposure of the autoradiogram in fig.2C–E and G reveals hybridizing bands both above and below the main bands (not shown). These results indicate a higher accumulation of α- and β-tubulin mRNA in epimastigotes than in metacyclic trypomastigotes.

3.3. *The homologous DNA clone λ Tc3 possesses DNA sequences encoding α- and β-tubulin genes*

In order to show that the 11.5 kb DNA insert of λ Tc3 contains sequences homologous to both α- and β-tubulin genes the recombinant DNA was cut with the restriction enzymes *Alu*I and *Hae*III and hybridized to two heterologous probes pDmTα1 [18] (*D. melanogaster*) and pβ2 [19] (*Lyt. pictus*). The *Alu*I digest generated fragments of 1.7 kb and 0.8 kb which are hybridizable only to the α-tubulin probe, and a 1.1 kb fragment hybridizable only to

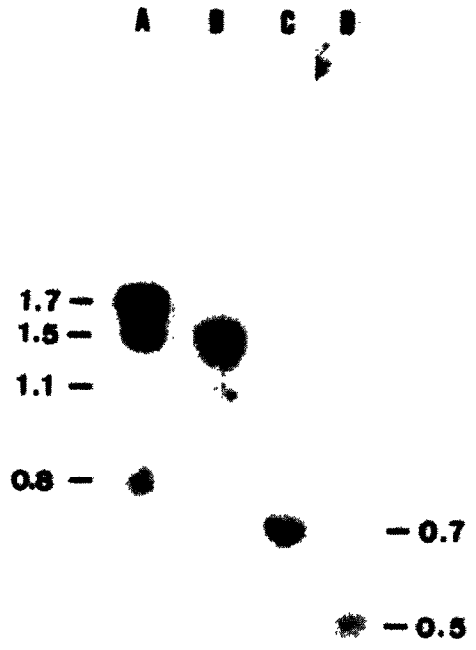

Fig.3. Hybridization of λ Tc3 DNA fragments, generated by digestion with *Alu*I and *Hae*III, with pDmTα1 and pβ2 probes. The digests were separated on a 1% agarose gel and transferred to nitrocellulose filter. *Alu*I digests (A and B); *Hae*III digests (C and D). A and C were hybridized with pDmTα1; B and D with pβ2. Numbers indicate the size in kilobases of the fragments.

β-tubulin probe. The 1.5 kb fragment hybridizes to both tubulin probes (fig.3A and B). The *Hae*III digests show a 0.7 kb fragment hybridizable to the α probe and a 0.5 kb fragment hybridizable to β probe (fig.3C and D). These results show that there are specific regions of the λ Tc3 cloned DNA that are hybridizable only to the α probe; the 1.7 kb and 0.8 kb *Alu*I fragments, and the 0.7 kb *Hae*III fragment, as well as β specific fragments; the 1.1 kb *Alu*I fragment and the 0.5 kb *Hae*III fragment.

4. DISCUSSION

The results above show that the α- and β-tubulins of *T. cruzi* are of ~ 55 kDa. Attempts to resolve the two subunits using low purity SDS were not successful (not shown). The hybridization of *T. cruzi* cytoplasmic RNA with the heterologous α- and β-tubulin probes revealed an α-tubulin mRNA of 1.6 kb and two β-tubulin mRNAs of 1.6 kb and 2.0 kb, respectively. The analysis of both in vivo pulse labelled proteins of epimastigote and metacyclic trypomastigotes, as well as the in vitro translation of equal amounts of their poly(A)$^+$ containing RNAs, revealed a higher level of synthesis of α- and β-tubulin in epimastigotes. The higher capability of epimastigote transcripts to synthesize in vivo and in vitro α- and β-tubulin is indicated to be due to a higher accumulation, or selective stabilization, of the α- and β-tubulin transcripts in the polysomal fraction of that form than in metacyclic trypomastigotes. The 4-fold higher hybridization signal of epimastigote tubulin mRNAs in both total cell RNA and polysomal poly(A)$^+$ RNA probed with λ Tc3 is compatible with a higher accumulation of those RNA species in epimastigotes than in metacyclic trypomastigotes. Furthermore, the tubulin transcripts are apparently confined to the polysomal compartment since they were not detected in the post-polysomal fraction of either epimastigotes or metacyclic trypomastigotes. Thus during metacyclogenesis there is a decrease in the accumulation of tubulin transcripts in polysomal poly(A)$^+$ RNA leading to a lower level of translatable tubulin mRNAs.

It has been shown in *Chlamydomonas reinhardtii* [25–28] that the control of tubulin gene expression is also operated through the modulation of the level of mRNA accumulation. The flagellar regeneration in that organism is accompanied by an increase in the amount of α- and β-tubulin mRNAs. These results were obtained by in vivo translation, Northern blot analysis and in vitro transcription in isolated nuclei. In contrast, in *L. mexicana* [3,4] the control of tubulin gene expression operates at a post-transcriptional level, as indicated by the fact that similar amounts of tubulin mRNAs were found in promastigote and amastigote cells.

Our results indicate that the control of tubulin gene expression during metacyclogenesis of *T. cruzi*, occurs primarily at the level of tubulin mRNA accumulation, although a post-translational control exerted by MAPs cannot be excluded. In another trypanosomatid [5], *L. enrietii*, it was found that the control of tubulin gene expression also operates at the level of mRNA accumulation during differentiation of promastigotes into amastigote cells.

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