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α- and β-tubulin mRNAs of *Trypanosoma cruzi* originate from a single multicistronic transcript

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The cluster of alternated α- and β-tubulin genes in the genome of *Trypanosoma cruzi* was shown to be transcribed into a single RNA molecule which upon processing gives rise to the mature α- and β-tubulin mRNAs. This conclusion was based on: (i) nuclear RNA species with the same molecular mass hybridize to both α- and β-tubulin cDNA probes; (ii) S_1 nuclease assay of the clustered tubulin genes has shown protected DNA fragments of the same size and of greater molecular mass than that corresponding to the mRNAs, hybridizable to both α- and β-tubulin cDNA probes; (iii) β-tubulin hybrid selected RNA is still able to hybridize to α-tubulin probe.

Tubulin gene; Multicistronic RNA;(*Trypanosoma cruzi*)

1. INTRODUCTION

In a previous work [1] we have shown that the tubulins, the major cytoskeletal protein, have their genes developmentally regulated during the operation of the *Trypanosoma cruzi* life cycle. The lowest level of tubulin mRNA accumulation and tubulin synthesis is seen in metacyclic trypomastigotes, the infective form of the organism. The tubulin gene organization in *T. cruzi* seems to be more elaborated than in *T. brucei* [2]. It comprises a basic 4.3 kb repeat unit of alternated α- and β-tubulin genes [3]. Besides this basic arrangement, units of linked α- and β-tubulin genes are also found in the genome of *T. cruzi* [3,4]. Furthermore, the tubulin gene family organization displays noticeable variation among several *T. cruzi* stocks and cloned lines analysed [3]. Besides the peculiar organization of the tubulin genes in trypanosomatids, the mRNA synthesis in these organisms and other kinetoplastids has been shown to present puzzling features like the addition of a common leader sequence (mini-exon) to the 5′ termini of all mRNAs, carried out by a process of trans-splicing [5–9]. Equally intriguing is the phenomenon called RNA editing of mitochondrial transcripts. It was shown that the mRNAs for the cytochrome-*c* oxidase subunit II and III contain uridine residues that are not encoded by the respective genes [10,11]. Another unique feature of the RNA metabolism of trypanosomatids is the occurrence of multicistronic transcripts comprising different mRNA units: variant surface glycoprotein 221-a mRNA and seven other stable mRNAs [12,13]; α- and β-tubulins mRNAs [14]; phosphoglycerate kinase

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mRNAs [15], all in *T. brucei*; α-tubulin mRNA in *Leptomonas seymouri* [16] and the IF-8 multigenic unit in *T. cruzi* [17]. In the majority of the cases the multicistronic RNA precursor was not clearly detected in Northern blots; one exception is the RNA from the IF-8 unit in *T. cruzi*. Here, we present evidence for the occurrence of a stable and common multicistronic transcript comprising α- and β-tubulin mRNAs in *T. cruzi*.

2. MATERIALS AND METHODS

2.1. *Cultivation of* T. cruzi

Epimastigote cells of the clone 14 isolated from the CL strain of *T. cruzi* [18] were maintained in liver infusion medium (LIT) at 29°C [19]. For all experiments, 2-day-old exponentially growing cultures containing 6.0×10^7 parasites/ml were used.

2.2. *Construction of the cDNA library*

Construction of the cDNA library, using the phage λgt11 as vector, was according to Dobner et al. [20]. Briefly, double-stranded cDNA was synthesized from 10 μg epimastigote polysomal poly(A^+) RNA, digested with S_1 nuclease, and modified by *Eco*RI methylase. After addition of *Eco*RI linkers and digestion with *Eco*RI, the cDNA was size-fractionated on 1% low-melting-temperature agarose gel. Fragments greater than a 580-base-pair marker were recovered from the agarose by extraction with butanol containing hexadecyltrimethylammonium bromide and further purified by spermine precipitation. The final cDNA preparation was inserted into the *Eco*RI site of the λgt11 bacteriophage. DNA was packed in vitro using a commercial extract (Stratagene), and portions of the reaction mixture were used to infect *Escherichia coli* strain Y1088. α- and β-tubulin cDNA clones were identified and purified from this library using a human α-tubulin cDNA clone (pspKα1) [21] and a sea urchin β-tubulin cDNA clone (pJ1) [22], which were gifts from Drs P. Dobner and M. Nemer, respectively.

2.3. *In vitro labelling of DNA clones*

DNA clones were labelled in vitro by nick-translation using [^{32}P]dATP [23]. The nick-translated DNAs were separated from non-incorporated dATP by Sephadex G-50 spin column chromatography [24]. The tubulin clones used were: a genomic clone, isolated from our library, called λTc3, a 11.5 kb *Eco*RI DNA fragment containing alternated α- and β-tubulin sequences [1]; pTcα3 (1.9 kb insert) and pTcβ4 (0.7 kb insert) DNA clones isolated from our cDNA library. The cloned DNAs were prepared according to Maniatis et al. [24]. The cDNA clones, originally cloned in the *Eco*RI site of λgt11, were subcloned in pUC18.

2.4. *RNA isolation*

Total, nuclear, and cytoplasmic RNA from epimastigote cells were phenol extracted in a buffer containing 7 M urea, according to Holmes and Booner [25], and purified by LiCl precipitation. Nuclei were isolated according to Wieben and Pederson [26].

2.5. *Nucleic acid electrophoresis, transfer to nitrocellulose filters and molecular hybridization*

Cloned DNAs, digested with *Eco*RI, were analysed in 0.8% agarose gels in TBE buffer as in [24]. The DNA fragments were double-transferred to nitrocellulose filters after denaturation and neutralization as described by Southern [27]. Total, cytoplasmic, and nuclear RNAs were denatured by the formamide-formaldehyde method and size fractionated on 1.2% agarose-formaldehyde gels [28]. The RNAs were transferred to nitrocellulose filters as described by Thomas [29]. Hybridizations were performed in high excess of nick-translated probes under high stringency conditions (50% formamide, 100 μl/ml denatured salmon sperm DNA, 5 × SSC, 5 × Denhardt solution and 50 mM Na_2HPO_4 buffer (pH 7.0), at 45°C). The filters were also washed under high stringency conditions (0.1 × SSC, 0.1% SDS at 50°C). Isolation of the homologous cDNA clones, using the heterologous human α-tubulin and sea urchin β-tubulin clones was also performed under the stringency conditions described above. The filters were autoradiographed with Kodak X-ray film, using an intensifying screen (lightning plus, Du Pont) at −70°C.

2.6. *S_1 nuclease mapping*

Total RNA (50 μg) was hybridized to 1.0 μg of previously denatured λTc3 genomic clone in a buffer containing 80% formamide as described [24]. After hybridization for 3 h at 55°C, ice-cold S_1 buffer, containing 100.0 U/ml of S_1 nuclease and 20 μg/ml of denatured salmon sperm ssDNA, was added and the mixture incubated at 37°C for 30 min. The samples were phenol extracted and precipitated with 20 μg carrier yeast tRNA by addition of 2.5 vols ethanol. The pellet was resuspended in alkaline buffer and the material submitted to electrophoresis in a 1.2% alkaline agarose gel as in [24]. After neutralization the material was transferred to nitrocellulose filters and hybridized separately to nick-translated α- and β-tubulin cDNA clones.

2.7. *Sandwich hybridization*

DNA dots of 0.5 μg β-tubulin cDNA clone insert (pTcβ4) were prepared as in [24]. The DNA dots were then prehybridized in buffer I (50% formamide, 0.75 M NaCl, 0.1 M Pipes, pH 6.4, 8 mM EDTA, 0.5% of 50 × Denhardt solution, 100.0 μg yeast tRNA, 0.5% SDS) for 18 h at 43°C. After prehybridization, the filters were washed 10 times in buffer II (1 × SSC, 2 mM EDTA, 0.5% SDS) at 60°C. The filters were hybridized to 60.0 μg total cell RNA in buffer III (50% formamide, 0.4 M NaCl, 10 mM Pipes, pH 6.4, 8 mM EDTA, 0.5% SDS) for 24 h at 43°C [30]. After 10 washes in buffer IV (1 × SSC, 0.5% SDS) at 50°C and 5 washes in the same buffer without SDS the filters were hybridized to nick-translated α-tubulin cDNA insert (pTcα3) and washed as described for Northern blots. A control β-tubulin DNA dot, not prehybridized to total RNA, was also hybridized in the same bag.

3. RESULTS

3.1. *Northern blot analysis of tubulin RNA transcripts of* T. cruzi

In order to identify the tubulin RNA species of *T. cruzi*, total RNA was hybridized to 3 different clones: a homologous genomic clone (λTc3) en-

coding α- and β-tubulin [1] and to homologous α- and β-tubulin cDNA clones (pTcα3, pTcβ4). Fig.1A shows that λTc3 recognized several RNA species ranging from 1.6 to approx. 20.0 kb of the cDNA clones, pTcβ4 (fig.1B) recognized mRNA bands of 1.6, 2.1 and 2.4 kb; whereas pTcα3 (fig.1C) hybridized to a 2.1 kb mRNA species that co-migrates with one of the β-tubulin mRNAs. Furthermore, both cDNA clones recognized the same higher molecular mass RNA transcripts detected by the genomic clone. One of the high molecular mass RNAs, the 6.0 kb species, gave a weaker signal than the other RNA bands upon hybridization with the cDNA probes. These results indicate that the 1.6, 2.1 and 2.4 kb transcripts are mature β-tubulin RNAs and one of the 2.1 kb transcripts is the mature α-tubulin RNA. The high molecular mass RNAs of 6.0, 8.0, 13.0 and 20.0 kb possibly represent intermediate processing products of the tubulin mRNA precursor.

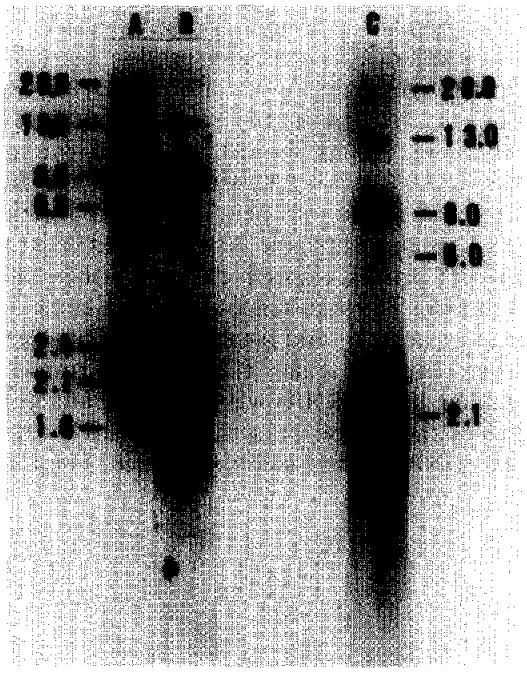

Fig.1. Analysis of tubulin RNA in epimastigote cells. 5 μg total RNA were denatured as described in the text and size separated on a 1.5% (A,B) or 1.2% (C) formamide-formaldehyde agarose gel. After transfer to nitrocellulose filters RNAs were hybridized to nick-translated λTc3 genomic cloned DNA (A), pTcβ4 (B) and pTcα3 cDNA clones (C). Numbers indicate the sizes (in kb).

3.2. *Compartmentalization of the multiple tubulin RNA species*

The nuclear and cytoplasmic tubulin RNA species were identified by their distribution on these cellular compartments. In this experiment total, cytoplasmic and nuclear RNAs were extracted from epimastigote cells and analysed on Northern blots and then hybridized to the λTc3 genomic cloned DNA. The hybridization of the total RNA revealed the same RNA bands depicted in fig.1. The α- and β-tubulin mRNA species of 2.4 and 2.1 kb were observed to be confined to the cytoplasmic compartment (fig.2B). The β-tubulin mRNA species of 1.6 kb can be seen on longer exposure of the autoradiograph (not shown). The high molecular mass RNA species of 20.0, 13.0, 8.0, and 6.0 kb were restricted to the nuclear compartment (fig.2C).

3.3. *S_1 nuclease protection of λTc3 genomic cloned DNA by total cell RNA*

In order to distinguish whether the high

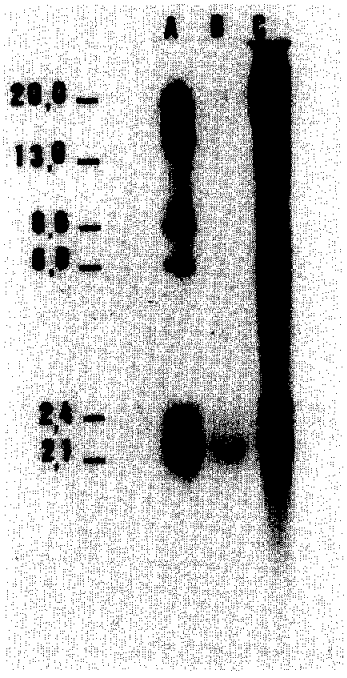

Fig.2. Compartmentalization of tubulin RNA species. 5 μg total and nuclear RNAs (A,C) and 2.0 μg cytoplasmic RNA were treated as described in fig.1 and hybridized to nick-translated λTc3 genomic cloned DNA. Total cell RNA (A), cytoplasmic RNA (B) and nuclear RNA (C). Numbers indicate kb.

molecular mass α- and β-tubulin hybridizable sequences were contained in a single or different RNA molecules, S_1 nuclease protection assay was performed. The genomic clone λTc3, hybridizable to α- and β-tubulin RNAs, was hybridized to total cell RNA and treated with S_1 nuclease as described in section 2. As depicted in fig.3, total RNA was able to protect λTc3 DNA fragments hybridizable to both α- (fig.3B) and β-tubulin (fig.3C) specific cDNA probes. The size of the protected fragments covers the region from approx. 2.0 to 10.0 kb, thus encompassing the range of magnitude of the mRNAs and that of the putative tubulin RNA precursors.

3.4. *β-Tubulin cDNA clone is able to hybrid select RNA hybridizable to α-tubulin cDNA probe*

The existence of contiguous RNA transcripts coding for α- and β-tubulin in *T. cruzi* cells was further examined by a 'sandwich experiment'. β-Tubulin cDNA clone insert, from pTcβ4, was immobilized onto nitrocellulose filter discs and hybridized to a large excess of total RNA. The filter was washed as described in section 2 and then hybridized to nick-translated α-tubulin cDNA insert from pTcα3. Fig.4B shows that the filter containing β-tubulin hybrid selected RNAs was still able to hybridize to the α-probe in comparison to the control filter not hybridized to total RNA (fig.4A). This result indicates the occurrence of single RNA transcripts, containing sequences for both α- and β-tubulin, in *T. cruzi* cells.

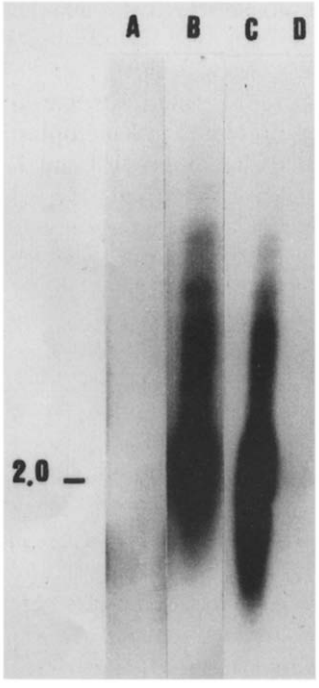

Fig.3. S_1 nuclease protected fragments of λTc3 genomic cloned DNA by total cell RNA. 50 μg total cell RNA were hybridized to 1 μg denatured λTc3 DNA, treated with S_1 nuclease, size fractionated on a 1.2% alkaline agarose gel, transferred to nitrocellulose filter and hybridized to nick-translated pTcα3 (A,B) and pTcβ4 (C,D) cDNA clones. λTc3 DNA (A); λTc3 DNA + total RNA (B); λTc3 DNA + total RNA (C) and λTc3 DNA (D).

3.5. *Two different restriction fragments of a single cDNA clone insert are able to hybridize to either α- or β-tubulin cDNA probes*

A clone isolated from our cDNA library (λTcα/β), using a human α cDNA clone as a probe (pspKα1), was shown to produce two fragments upon digestion with *Eco*RI when hybridized to itself (fig.5A). The 0.9 kb fragment was shown to be hybridizable to pTcα3 cDNA clone (fig.5B) and to the human α-tubulin probe pspKα1 (not shown), but not to β-tubulin probe. The 0.8 kb fragment hybridized to the pTcβ4 cDNA clone (fig.5C) as well as to the sea urchin β-tubulin cDNA clone pJ1 (not shown), but not to α-tubulin probe. The 0.9 and 0.8 kb fragments of the clone λTcα/β, subcloned into pUC18, yielded a pattern of RNA bands identical to that produced by pTcα3 and pTcβ4 probes, respectively, on Northern blots (not shown). The occurrence of a cDNA clone containing separate α- and β-tubulin hybridizable sequences suggests that a precursor transcript containing both mRNAs was primed by oligo(dT), copied by the reverse transcriptase during the first-strand cDNA synthesis and then cloned. This inter-

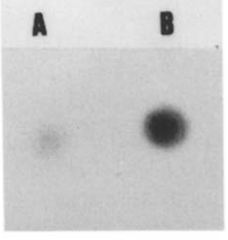

Fig.4. Sandwich hybridization experiment. DNA dots of 0.5 μg pTcβ4 cDNA insert were first hybridized to 60 μg total RNA followed by a second hybridization to nick-translated pTcα3 insert DNA as given in text. pTcβ4 × pTcα3 (control) (A); pTcβ4 previously hybridized to total RNA × pTcα3 (B).

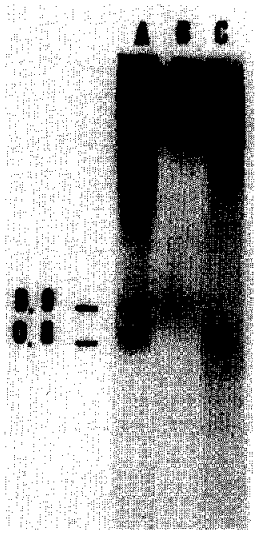

Fig.5. Two different restriction fragments of a single cDNA clone insert is able to hybridize to α- and β-tubulin cDNA probes. The recombinant cDNA clone λTcα/β was digested with *Eco*RI and size fractionated on a 0.8% agarose gel. After transfer to nitrocellulose the cloned DNA fragments were hybridized to nick-translated λTcα/β DNA (A); pTcα3 DNA (B) and pTcβ4 DNA (C). Numbers indicate size of *Eco*RI fragments in kb.

pretation is further supported by the observation that all high molecular mass tubulin RNA species are polyadenylated (not shown).

4. DISCUSSION

Here, we have reported data showing that the α- and β-tubulin mRNAs of *T. cruzi* originate from a unique multicistronic precursor RNA, transcribed from the cluster of the tandemly repeated α- and β-tubulin genes. As described by Moura-Neto [4] and Maingon et al. [3], the tubulin genes in *T. cruzi* are linked and tandemly repeated in the genome of the parasite. Maingon describes a basic repeat unit of alternated α- and β-tubulin genes of 4.3 kb. One of the clones studied by that author contains four tandemly repeated units of length 4.3 kb, together totalling 17.2 kb. Thus, such a segment of genomic DNA is large enough to produce transcripts of size similar to those detected in our Northern blots.

The following results, obtained here, constitute strong evidence of the existence in *T. cruzi* cells of multicistronic precursor transcripts that, upon processing, give rise to the mature α- and β-tubulin mRNAs: (i) the nuclear localization of the high molecular mass tubulin RNA species (6.0–20.0 kb); (ii) the resistance to S_1 digestion of fragments of the genomic clone λTc3, with molecular mass higher than that of the mature tubulin mRNA species, and (iii) the ability of β-tubulin hybrid-selected RNA to hybridize to α-tubulin probe (sandwich experiment). Furthermore, the finding of the cDNA clone (λTcα/β) with an insert containing two *Eco*RI fragments, one hybridizable to α- and the other to β-tubulin probes, is consistent with the idea of a multicistronic RNA copied by the AMV reverse transcriptase during cloning. Although the library was constructed from polysomal poly(A^+) RNA, a small amount of nuclear leakage or lysis may have occurred, since Northern blots of such RNA preparations show discrete contamination of the abundant 6.0 and 8.0 kb RNA species (not shown). Furthermore, all the high molecular mass RNA species were shown to be polyadenylated (not shown). Therefore, these RNAs can be primed by oligo(dT) during cDNA synthesis. The λTcα/β-tubulin cDNA clone is presently being studied in more detail in our laboratory.

A description of multicistronic transcription units has been presented in Kinetoplastidae like *T. brucei* [12,13], *L. seymouri* [16] and *T. cruzi* [17]. In *T. brucei* and *L. seymouri* [12,16] the occurrence of primary transcripts from multicistronic DNA units has been shown by analysis of nascent transcripts. However, in many cases it was not possible to identify clearly the precursors in the steady-state RNA population [14,16]. In *T. cruzi* evidence for stable multicistronic precursors has been presented, initially for the IF-8 multigenic unit [17], and now also for the tubulin gene cluster. The high level of accumulation of tubulin RNA precursor appears as an attractive model for studying the unusual mechanism of RNA processing in trypanosomatids.

The difficulty in detecting multicistronic precursors in steady-state RNAs indicates rapid processing of the primary transcript as described for *T. brucei* [14] and *L. seymouri* [16]. In *T. cruzi*, in contrast, the multicistronic transcripts described, i.e. the IF-8 [17] and now tubulin, are rather stable and accumulate to high levels. This circumstance may reflect post-transcriptional control of this coordinately expressed gene family in *T. cruzi* as described for PGK genes in *T. brucei* [17]. In fact,

as we have previously shown, tubulin gene expression is developmentally regulated during differentiation of *T. cruzi* [1]. The control of tubulin gene expression is being studied in our laboratory in epimastigotes, in the non-dividing metacyclic trypomastigotes and spheromastigotes generated in vitro as described by Rondinelli et al. [31]. Recent data (to appear elsewhere) show that in metacyclics, the decrease in level of mature mRNAs species, previously reported [1], is accompanied by an increase in amount of the 20.0 kb RNA species. Furthermore, in stationary and heat-shocked epimastigote cells this RNA species also accumulates. This accumulation is promptly reversed when cells progress towards lag phase and when the temperature is lowered to 29°C, respectively. Similarly, Muhich and Boothroyd [32] have also shown in *T. brucei* that the multicistronic tubulin transcripts are also induced to accumulate by heat shock. These observations suggest that under restricted conditions of cell division (metacyclic trypomastigotes, stationary and heat-shocked epimastigotes) a limited capacity for processing the putative 20.0 kb precursor RNA occurs. This may be considered to be an indication of the expression of tubulin genes, during differentiation of *T. cruzi*, being post-transcriptionally controlled.

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