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Transcription of the intergenic regions of the tubulin gene cluster of *Trypanosoma brucei*: evidence for a polycistronic transcription unit in a eukaryote

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ABSTRACT

The tubulin genes of T. brucei are clustered in a tightly packed array of alternating α- and β-genes. The steady state mRNA contains one abundant mRNA species each for α- and β-tubulin, both carrying the identical 35 nt mini-exon sequence at their 5'-ends.

We have used in vitro run-on transcription assays to investigate the mechanism of tubulin gene transcription in T. brucei. Our results show that the regions between the individual tubulin genes are transcribed at the same rate as are the genes themselves. On the other hand, transcripts containing the intergenic regions could not be detected by Northern analysis or in vivo labelling experiments. We conclude that putative transcripts from the intergenic regions have a half-life of less than one minute. These results suggest that the tubulin gene cluster is transcribed as a single contiguous transcription unit yielding a primary transcript which is rapidly processed into individual mRNAs by the polyadenylation and mini-exon trans splicing machineries.

INTRODUCTION

The parasitic hemoflagellate Trypanosoma brucei possesses a membrane skeleton and a flagellar axoneme as the two mayor microtubule-based cytoskeletal structures. The architecture and biochemistry of these structures remain unchanged throughout the complex life-cycle of the organism (1). Tubulin genes, which encode the predominant components of microtubules, can therefore be considered as bona fide household genes.

In T. brucei, tubulin genes are arranged as a tightly packed cluster of alternating α- and β-genes with a basic repeat length of 3.7 kb (2,3).This cluster, which has a maximal length of about 40 kb (4), was shown to be the locus of tubulin gene transcription. S1-mapping experiments with mature tubulin mRNA have demonstrated that the individual tubulin genes of the cluster are separated from each other by very short intergenic regions of about 170 bp (4). Mature α- and β-tubulin mRNA both carry an identical 35 nt mini-exon sequence at their 5'-termini (4,9), in analogy to what has been

found with other trypanosomal mRNAs (5,6,7,8). This mini-exon sequence is not encoded in the tubulin gene repeat nor within at least 12 kb upstream of the 5 -terminus of the tubulin cluster (4). Rather, 35 nt mini-exon sequences are coded for by a family of tandemly repeated 1.35 kb segments (10,11, 12), from which an abundant 140 nt RNA is transcribed (medRNA: mini-exon derived RNA), which carries the 35 nt mini-exon at its 5 -terminus (13,14, 15). These and other observations suggest that the mini-exon and the main-exons (e.g. tubulin-exons) are discontinuously transcribed (reviewed in 16). Different models have been proposed to explain this fusion of the mini-exon sequences to the main-exon sequences (5,13,15). Several indications favored the idea that the two exon sequences are combined by an intermolecular splicing reaction (16). Interestingly, a U2 snRNA analogue from trypanosomes has recently been identified (18). This RNA species, which is involved in cis splicing of eukaryotic pre-mRNA (19), may also fulfil a similar function in the processing of trypanosomal pre-mRNAs by trans splicing. A potential intermediate of the theoretical trans splicing model, a Y branch structure, has been found in trypanosomal steady state RNA (20,21, 22). Hence, it is now generally believed that trypanosomes join mini-exon sequences to main-exon sequences by a trans splicing event.

The recently reported hsp70 genes of T. brucei (23) are the only published examples of protein-coding genes, where the whole transcription units have been identified. Transcription of these tandemly repeated genes seems both to initiate and to terminate within the short intergenic flanking regions. However, with the exception of a sequence showing a high homology to eukaryotic heatshock elements (24), homologies with other regulatory sequences are less obvious in the intergenic regions of trypanosomal hsp70 genes. The functional significance of the suspected hsp70 TATA-box, which is also found at an appropriate distance to the initiation site of mini-exon sequences in T. brucei (13), still remains to be elucidated.

In contrast to these heat-shock genes, the transcription units of the household genes studied so far, as well as of VSG genes, appear to span several protein-coding regions (16). In this study, we report the results of an analysis of the transcription unit of tubulin genes in T. brucei. Our results suggest that the entire tubulin gene cluster consists of a single transcription unit. The nascent transcript is immediately processed into individual mature mRNA molecules. Therefore, a polycistronic transcript may in fact never exist as a physical entity.

MATERIAL AND METHODS

Trypanosomes. Procyclic trypanosomes of either stock STIB 366 or stock 427 (25) were grown at 28° C in SDM-79 medium (26), supplemented with 10% fetal serum and 2ml of sterile hemin solution (2.5mg/ml in 50 mM NaOH) per liter.

Plasmids and vectors. The following plasmids have been described elsewhere: pCL102 (14), pTcV 121.7 (27), pTcPGK8 (28), pTgTIM12 (29), pR4 (30) and pTBtu9A (2). pEP-30 and pEP-40 vectors (P.W.L. unpublished results) were constructed by ligating the 514bp Rsa I fragment of pEMBL-9 (31), which contains the F1 origin, into the Pvu II site of pGEM-3 and pGEM-4 (Promega), respectively. These pEP constructs therefore allows the isolation of single stranded DNA upon superinfection with F1 phages (32).For producing inter- and intragenic subclones of the trypanosomal tubulin basic repeat, the plasmid pTBtu9A was appropriately digested and the eluted fragments were ligated into the polylinker region of pEP-30 or pEP- 40. In detail, the Taq I [positioned at nucleotide 3581 according to Kimmel et al. (44)] to Sau 96I [90] fragment and the Taq I [2305] to Taq I [2492] fragment of pTBtu9A were ligated into the Acc I site of pEP-40, yielding the intragenic subclones $pTBtu\beta_{160}$ and $pTBtu\alpha_{189}$, respectively. The intragenic subclone $pTBtu\beta_{222}$ was constructed by subcloning the Sau 3A [1032] to Sau 3A [1249] fragment of pTBtu9A into the Bam HI site of pEP-40. The intergenic subclones $pTBtu^{3'}\beta\text{-}5'\alpha$ and $pTBtu^{3'}\alpha\text{-}5'\beta$ were constructed by subcloning the Sau 3A [1696] to Sau 3A [1853] fragment and the Sau 96I [3419] to Taq I [3580] fragment of pTBtu9A into the Bam HI site of pEP-40 and pEP-30, respectively. The nucleotide sequence of the subcloned fragments was then verified by dideoxy chain-termination sequencing (33) using ^{35}S-labelled ATP. A summary of these subclones is given in Fig.1.

Isolation and blotting of nucleic acids. Total RNA, which was isolated from trypanosomes using the guanidinium/ isothiocyanate procedure (34), was size-fractionated on formaldehyde containing agarose gels (35) and transferred to nitrocellulose filters (36). The RNA blots were hybridized with nicktranslated DNA probes as previously described (4) and finally washed in a solution of 0.3X SSC / 0.1% SDS at 65° C for 40 minutes. Plasmid DNA was isolated according to the PEG-method (37) and was finally banded on a cesium chloride ethidium bromide equilibrium density gradient. Purified DNA was either digested with restriction enzymes, size-fractionated on agarose gels and then transferred onto nitrocellulose filters, or was denatured and spotted onto nitrocellulose filters with a filtration manifold according to

the manufacturer's instructions (Schleicher and Schuell). Hybridization of blotted DNA with ^{32}P-UTP labelled RNA was done in 5X SSC / 10% dextran sulfate / 0.2% SDS / 100 µg/ml depurinized CT DNA / 30 µg/ml yeast carrier RNA at 65° C for 12 hours. Final post-hybridization washes were done in 0.3X SSC / 0.1% SDS at 65° C for 15 minutes.

Labelling of nascent RNA. In vitro labelling involves the extension of nascent RNA in isolated nuclei of procyclic trypanosomes in the presence of α-^{32}P UTP. Briefly, nuclei of procyclic trypanosomes were prepared by N_2 gradient consisting of 15%, 25% and 50% of sucrose dissolved in lysis buffer. Purified nuclei were stored at a concentration of $10X10^9$ nuclei per ml of nuclei storage buffer (39) at -70° C. For a nuclear runon assay, nascent RNA in $2X10^9$ nuclei was elongated at 28° C for 8 minutes, and the labelled RNA was then purified and analyzed on DNA blots as described (40). In vivo labelling of nascent RNA was accomplished by growing procyclic trypanosomes of strain 427 in the presence of [^{3}H]-labelled adenosine as described (20). Duplicate cultures of trypanosomes ($1X10^7$ cells/ml) were incubated in the presence of 220 µCi/ml [2,5',8-^{3}H] adenosine (42-50 Ci/mmol, Amersham International) at 28° C in SDM-79 medium (26). At each time point, aliquots of 2 ml were taken and trypanosomes were harvested by a short centrifugation. Afterwards, cells were lysed in the presence of 0.1% SDS and 500 µg/ml Proteinase K at 50° C for at least one hour. Phenol-extracted and EtOH-precipitated nucleic acids were then dissolved in 5 mM $MgCl_2$ and 2 mM $CaCl_2$, and DNA was digested with 35 µg RNase free DNase I per ml (41). After several phenol-extractions and an ethanol precipitation, RNA was analyzed by hybridization to appropriate DNA probes immobilized on nitrocellulose filters (20). Nonspecifically bound DNA was removed by washing the filters in 1XSSC at 65° C for 30 minutes. Filter bound radioactivity was then counted in scintillation fluid (Insta-gel, United Technologies Packard, USA).

RESULTS

Analyses of tubulin mRNA by Northern blot hybridization

Earlier hybridization experiments demonstrated the abundance of mature tubulin mRNA, but had given no evidence for the existence of precursor RNA molecules. In these previous experiments tubulin encoding sequences were used for hybridization. In the present study, the possible existence of tubulin mRNA precursors was reinvestigated by using inter enic hybridization probes. These are short DNA sequences of about 160 bp which are derived from regions between individual tubulin genes (see Fig.1) and which are not present in

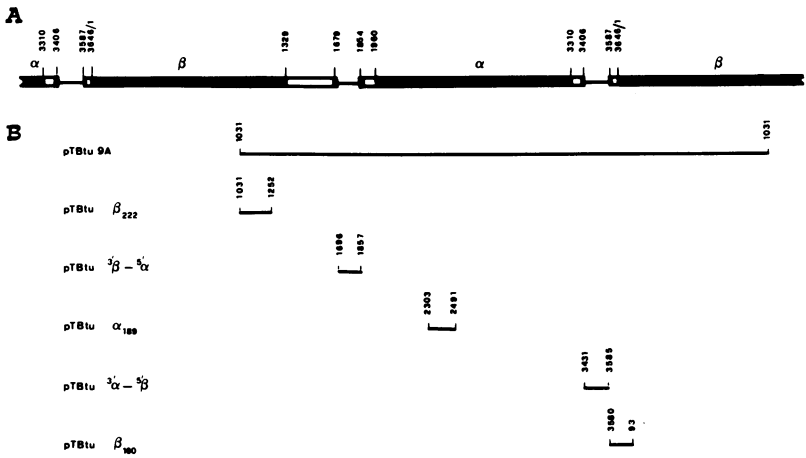

Fig. 1

A) A section of the tubulin gene cluster is shown. Translated (solid blocks) as well as untranslated, but transcribed (open blocks) regions of the tubulin gene cluster are depicted and numbered according to the sequence of Kimmel et al. (44). Fine lines denote regions of the cluster which are not present in mature tubulin mRNAs (4), and thus are called intergenic regions.

B) The size and position of tubulin subclones used in this study are denoted.

The tubulin gene cluster was dissected by subcloning different regions of comparable length from the plasmid pTBtu9A which contains a single repeat unit of the whole tubulin gene cluster. Except for the plasmid pTBtu 9A, which has been described previously, all other subcloned regions have been sequenced and therefore can exactly be positioned according to the sequence of Kimmel et al. (44). In this study, pTBtuα189, pTBtuß222 and pTBtuß160 are referred to as intragenic subclones, and pTBtu$^{3'}$α-$^{5'}$ß and pTBtu$^{3'}$ß-$^{5'}$α as intergenic subclones.

the mature tubulin mRNAs, as determined earlier by S1-mapping experiments (4). Total trypanosomal RNA was size-fractionated on an agarose gel containing formaldehyde, and blotted onto a nitrocellulose filter. Strips of such blots were then hybridized with probes representing either intergenic or intragenic regions (Fig.1B). The results presented in Fig.2 demonstrate that hybridization with intragenic regions detects the expected strong bands of α- and ß-tubulin mRNA. In contrast, no hybridization signal is found when the strips are probed with intergenic regions. These observations clearly confirm our earlier S1-mapping experiments (4) and demonstrate that no intergenic sequences of the tubulin gene cluster can be detected in steady-state RNA by Northern blot analyses.

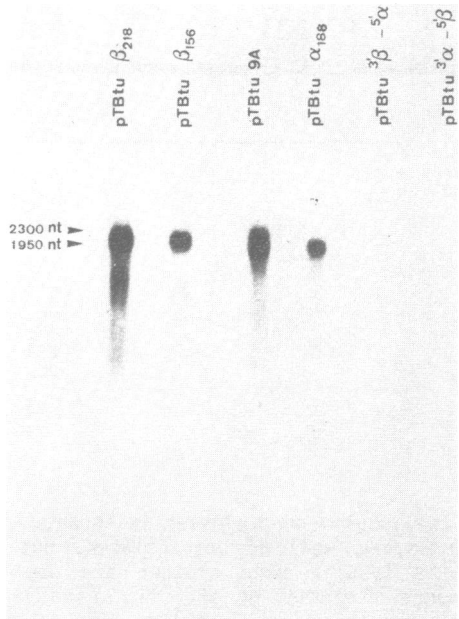

Fig. 2
Hybridization of intergenic and intragenic subclones from the tubulin gene cluster with trypanosomal RNA
Total RNA (5 µg) from procyclic trypanosomes was fractionated on a formaldehyde-containing agarose gel and transfered onto a nitrocellulose filter. Individual strips of the filter were hybridized with nicktranslated tubulin subclones as indicated. Posthybridization washes were done as described in Material and Methods, and the strips were exposed for 14 days. This results in heavy overexposure for the mature tubulin mRNAs but would have allowed the detection of minor RNA bands.
The size of mature α- and ß-tubulin mRNA has previously been described (4) and is indicated to the left.

Intergenic tubulin sequences are transcribed in vitro

The absence of intergenic sequences in stable RNA can result either from the fact that these regions are not transcribed or, alternatively, that these sequences are transcribed, but that the nascent transcript is rapidly processed and the intergenic sequences are eliminated. Hence, the transcription status of these intergenic regions was explored in run-on transcription experiments. Nuclei from procyclic trypanosomes were isolated, and nascent RNA chains were elongated in vitro in the presence of ^{32}P-UTP for 8 minutes. Size analysis of this RNA revealed that the transcripts are very short (20-30 nt), indicating that only a few nucleotides are added to the preexisting nascent RNA during the in vitro reaction (M.A.I., unpublished

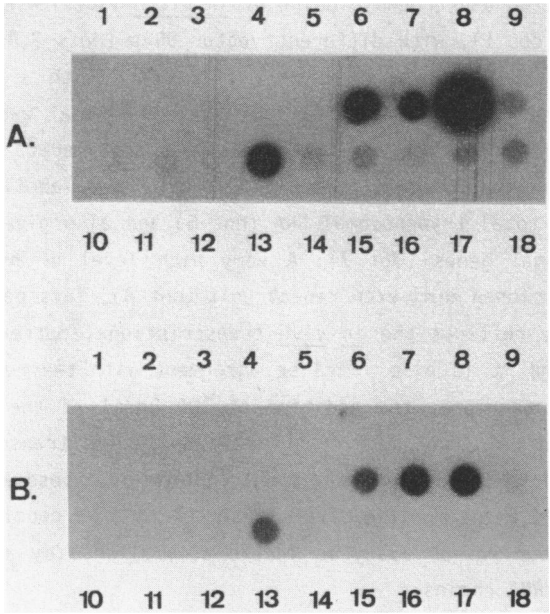

Fig. 3

Analyses of in vitro run-on transcripts by dot blot hybridization.

A) Nascent RNA was elongated in isolated trypanosomal nuclei from $2X10^9$ cells using α-^{32}P-UTP as described (40). Labelled RNA was then analyzed by hybridization to dot blotted DNA samples (5 ug) of different origin: 1) blank; 2) plasmid DNA of pBR322; 3) genomic DNA of E. coli JM107; 4) plasmid DNA of pEP-40; 5) plasmid DNA of pAT153; 6) genomic DNA of trypanosomes of stock STIB 366; 7) plasmid DNA of pR4, which contains ribosomal sequences of trypanosomes; 8) plasmid DNA of pCL102, which contains mini-exon sequences; 9) plasmid DNA of an unidentified trypanosomal gene; 10) plasmid DNA of pTcV121.7, which contains sequences coding for a variant surface glycoprotein; 11) plasmid DNA of pTcPGK8, which contains sequences encoding glycosomal phosphoglycerate kinase; 12) plasmid DNA of pTgTIM12, which contains sequences encoding glycosomal triose-phosphate isomerase; 13) plasmid DNA of pTBtu9A; 14) plasmid DNA of the intragenic subclone pTBtuβ_{160}; 15) plasmid DNA of the intergenic subclone pTBtu $^{3'}\alpha$-$^{5'}\beta$; 16) plasmid DNA of the intergenic subclone pTBtu$^{3'}\beta$-$^{5'}\alpha$; 17) plasmid DNA of the intragenic subclone pTBtuα_{189}; 18) plasmid DNA of the intragenic subclone pTBtuβ_{222}. Posthybridization washes were done as described in Material and Methods, and the blot was exposed for 14 days.

B) Run-on experiment as in A), but in the presence 100µg/ml of the RNA polymerase inhibitor α-amanitin. Analysis of labelled RNA was done by hybridization to an identical dot blot as described in A). Posthybridization washes were done as described in Material and Methods, and the blot was exposed for 14 days.

observations). Labelled RNA was then isolated and analyzed by hybridization to dot-blotted DNA .

A number of controls served to establish the reliability of the runon

system (Fig.3A). No background binding of in vitro labelled RNA was detected in a blank dot (dot 1), with different vector DNAs (dots 2,4 and 5) or with E.coli DNA (dot 3). No hybridization was detected with a variant surface glycoprotein gene (dot 10), while a low level of signal was detected with two genes coding for the glycolytic enzymes phosphoglycerate kinase (dot 11) and triose-phosphate isomerase (dot 12). The in vitro labelled RNA hybridizes strongly to total trypanosomal DNA (dot 6) and also gives a strong signal with ribosomal genes (dot 7). A very high level of hybridization is obtained with a cloned mini-exon repeat unit (dot 8). This pattern of hybridization clearly reflects the in vivo transcriptional pattern of procyclic trypanosomes, and it is also in close agreement with the results of Kooter et al. (41). Furthermore, the addition of 100 µg/ml of the RNA polymerase inhibitor α-amanitin (Fig. 3B) drastically abolishes transcription by RNA polymerase II (dots 11-18), whereas transcription of ribosomal genes (dot 7) is not influenced by α-amanitin. From these controls we conclude that labelling of RNA in our run-on assay is indeed accomplished by accurate elongation of nascent RNA chains.

Analyses of intergenic (Fig.3A; dots 15,16) and intragenic (Fig.3A; dots 14,17 and 18) regions of the tubulin gene cluster with such in vitro labelled nascent RNA gave approximately equal hybridization intensities. The intensity of these hybridization signals corresponds to what would be expected for true transcription of these regions. The hybridization signals of the intergenic and intragenic subclones are about 25 times less intense than the signal obtained with plasmid pTBtu9A (Fig.3A; dot 13), which contains a whole tubulin repeat unit. This difference reflects the actual size difference between the 160 bp of the intergenic and intragenic regions and the 3.7 kb of the tubulin repeat unit. The results of these in vitro run-on experiments thus suggest that intergenic and intragenic regions are transcribed at the same rate.

Turnover of intergenic and intragenic tubulin transcripts

Since the run-on experiments indicated that the intergenic regions are transcribed, even though stable RNA from this region is not detectable, we next addressed the question of RNA turnover. RNA in procyclic trypanosomes was continuously labelled in vivo by growing trypanosomes in medium containing ^{3}H-adenosine (42). At different time points aliquots from the culture were collected and the trypanosomes were harvested. After isolation and purification of RNA, the in vivo labelled RNA was analyzed by hybridization

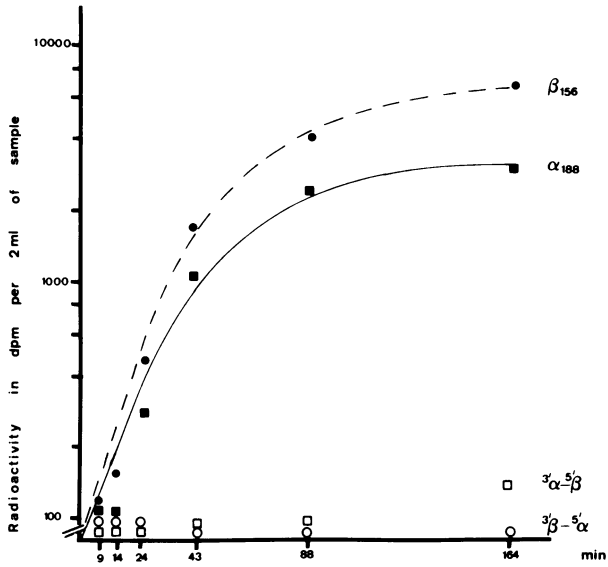

Fig. 4
Kinetics of in vivo labelled RNA from intergenic and intragenic regions of the tubulin gene cluster.
In vivo [^{3}H]-labelled RNA was isolated at the times indicated from duplicates of 2 ml culture aliquots and was analyzed by hybrid selection with either intergenic or intragenic subclones of the tubulin gene cluster as described in Material and Methods. Filter-bound radioactivity was determined by liquid scintillation counting, and the counted radioactivity of each time point was corrected for background hybridization to vector DNA and the duplicates were then averaged.

to different intergenic and intragenic fragments from the tubulin gene cluster. The labelling kinetics thus obtained are shown in Fig.4. These results demonstrate that in vivo labelled RNA hybridizing to either of the intragenic tubulin probes accumulate at similar rates and to similar steady-state levels. This is in perfect agreement with the results from run-on transcription, which had shown that both genes are equally transcribed. In contrast, the rate of accumulation and steady state level of RNA hybridizing to intergenic sequences is 100 times lower and thus within background. This vast difference in RNA accumulation, in conjunction with the above observations that intergenic and intragenic sequences are both transcribed with similar efficiency, strongly suggest that the RNA moieties transcribed from the intergenic regions of the tubulin gene cluster do indeed have a very short half-life.

DISCUSSION

This study describes the results of the transcription analyses of the tubulin gene cluster of Trypanosoma brucei. In isolated nuclei, intergenic regions are transcribed at a rate approximately equal to that of intragenic regions, whereas intergenic sequences are not detectable in steady-state RNA or in RNA labelled *in vivo*. It is unlikely that the transcription of intergenic sequences in isolated nuclei is an *in vitro* artefact caused by relocation of polymerases or by nonfunctional termination, since the nascent RNA is elongated by only a few nucleotides. An erroneous transcription of several intergenic nucleotides is unlikely to cause the observed hybridization signal. Our results do not rule out the possibility of very closely linked transcription termination and initiation signals within the intergenic regions, although any disruption in transcription would be expected to decrease the hybridization signal to DNA segments of such small size. The most straightforward interpretation of the run-on results is that RNA polymerase molecules are evenly distributed over the entire tubulin gene cluster. Nevertheless, transcripts containing intergenic sequences could not be detected in steady-state RNA or in RNA labelled *in vivo*. Given an equal rate of synthesis of inter- and intragenic sequences, the differences in steady-state levels must be due entirely to differences in half-lives. The *in vivo* labelling experiment shown in Fig.4 demonstrates that, within two and a half hours, RNA containing intragenic sequences accumulates to a level 100-fold higher than that of RNA containing intergenic sequences. This 100-fold difference in steady-state levels implies a similar difference in half-lives. The difference may even be greater if equilibrium of labelling has not been reached at this point. Assuming that labelling of RNA containing intragenic sequences is at or near equilibrium after two and a half hours, it can be concluded that half-maximal labelling is reached after about 75 minutes, which in a steady-state situation, implies a half-life of 75 minutes as well. This value falls within the range determined for the half-life of ß-tubulin mRNA by Ehlers et al. (42). A half-life of 75 minutes for RNA containing intragenic sequences implies a half-life of less than one minute for transcripts of the intergenic regions. If the tubulin gene cluster is transcribed as a single contiguous unit and at a rate of about 5 kb per minute as in other eukaryotes (43), then a half-life of transcripts from the tubulin intergenic stretches of less than one minute strongly suggests that processing of the primary transcript is co-transcriptional.

Polycistronic transcription units have also been suggested for other trypanosomal household gene clusters (16). In contrast to the situation for

tne tubulin gene cluster, in some of these other cases, putative precursors larger than the mature transcripts could be detected in steady-state RNA by Northern or S1 nuclease analyses (16,43). Whether or not precursor transcripts of a putatively polycistronic transcription unit are present at detectable levels likely depends on the rate of processing of the primary transcript. The rate of processing may depend on the processing of the primary efficiency of acceptor sites for the trans splicing reaction with the medRNA and may be an important point of regulation of the levels of mature mRNAs in trypanosomes.

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