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ORIGINAL ARTICLE

Life-Cycle and Growth-Phase-Dependent Regulation of the Ubiquitin Genes of *Trypanosoma cruzi*

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Background. *Trypanosoma cruzi*, the causative agent of Chagas disease, exhibits a complex life cycle that is accompanied by the stage-specific gene expression. At the molecular level, very little is known about gene regulation in trypanosomes. Complex gene organizations coupled with polycistronic transcription units make the analysis of regulated gene expression difficult in trypanosomes. The ubiquitin genes of *T. cruzi* are a good example of this complexity. They are organized as a single cluster containing five ubiquitin fusion (*FUS*) and five polyubiquitin (*PUB*) genes that are polycistronically transcribed but expressed differently in response to developmental and environmental changes.

Methods. Gene replacements were used to study *FUS* and *PUB* gene expression at different stages of growth and at different points in the life cycle of *T. cruzi*.

Results. Based on the levels of reporter gene expression, it was determined that *FUS1* expression was downregulated as the parasites approached stationary phase, whereas *PUB12.5* polyubiquitin gene expression increased. Conversely, *FUS1* expression increases when epimastigotes and amastigotes differentiate into trypomastigotes, whereas the expression of *PUB12.5* decreases when epimastigotes differentiate into amastigotes and trypomastigotes.

Conclusions. Although the level of CAT activity in logarithmic growing epimastigotes is six- to seven-fold higher when the gene was expressed from the FUS1 locus than when expressed from the *PUB12.5* locus, the rate of transcription from the two loci was the same implying that post-transcriptional mechanisms play a dominant role in the regulation of gene expression.

Key Words: Gene expression, Parasite, Transcription.

Introduction

*Trypanosoma cruzi i*s a protozoan hemoflagellate that causes Chagas disease in many countries of Central and South America. This disease has caused extensive morbidity and mortality throughout Latin America. The life cycle of *T. cruzi* is characterized by a succession of four biochemically and morphologically distinct developmental forms which allow the parasite to adapt to the different environments it encounters in the vertebrate and invertebrate host. In the insect vector, the epimastigote replicates in the midgut prior to differentiating into the non-replicative metacyclic trypomastigote in the hindgut. When introduced into the bloodstream of the mammalian host, the metacyclic trypomastigote invades cells and differentiates into the amastigote. Amastigotes multiply rapidly within cells and differentiate into bloodstream trypomastigotes, which when released from the cell can either initiate another round of infection or be taken up by the insect vector, thus completing the life cycle.

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Although the complex life cycle of *T. cruzi* is undoubtedly accompanied by the stage-specific expression of genes, very little is known about gene regulation in trypanosomes. A reason for this is that there are major differences between trypanosomes and higher eukaryotes in the organization of protein-coding genes and in the mechanisms of their expression. One difference is that all trypanosome protein-coding genes analyzed thus far are organized into polycistronic transcription units (1–6). Further, several groups have demonstrated that functionally unrelated genes can be part of the same transcription unit (7–9). In addition, many of these polycistronic transcription units contain genes which are transcribed from the same promoter but which yield very different steady-state RNA levels and/or are differentially expressed during the life cycle (10). This implies that differential expression of the various genes is probably mediated by post-transcriptional mechanisms.

The study of the mechanisms that regulate gene expression in trypanosomes is further complicated by the fact that many protein-coding genes are members of multicopy gene families, which are arranged frequently in tandem arrays, with short intergenic regions separating the protein-coding sequences (11–15). The presence of multiple gene copies makes the study of the regulated expression of individual members difficult.

The initial characterization of the ubiquitin loci of *T. cruzi* identified five genes that code for an ubiquitin-fusion protein (*FUS* genes) and five polyubiquitin genes (*PUB* genes) (14). The five *PUB* genes consist of varying numbers of the ubiquitin coding sequence followed by the non-ubiquitin extension that forms part of the *FUS* genes. Interestingly, the *PUB* and *FUS* genes are differentially expressed in response to different environmental stimuli (14). Further characterization described the linkage of the ubiquitin genes to upstream calmodulin genes and the organization of these genes within the genome into two unlinked loci (14–16). It has also been shown that the *CAL, CUB, FUS* and *PUB* genes are transcribed from the same DNA strand (14–16).

This report describes the differential expression of the ubiquitin genes of the *2.65* ubiquitin-calmodulin locus at different stages of growth and during the developmental cycle of *T. cruzi*. Gene replacements facilitated a comparison of the expression of *FUS1* and *PUB12.5* genes.

Materials and Methods

Culture Conditions

Trypanosoma cruzi strain CL epimastigotes were grown at 28°C in liver infusion tryptose medium (LIT) supplemented with 10% fetal calf serum and 0.1 mg/mL hemin (17). Following electroporation, transformed clones were isolated from G418-resistant *T. cruzi* populations by limiting dilution in the absence of drug selection (18,19).

NIH 3T3 cells grown to 50% confluence in DMEM supplemented with 2% fetal calf serum were infected with log-phase epimastigotes. The amastigotes and trypomastigotes released into the infected culture supernatant were harvested every other day at 3000 rpm/5 min (Heraeus multifuge 3S-R/3046 rotor). The amastigotes were separated from trypomastigotes using five pellet volumes of amastigotes specific antibody 2C2B6, specific for the Ssp-4 surface antigen of amastigotes (20) at 37°C for 1 h. The amastigotes were separated from trypomastigotes at 1000 rpm/1/min in a microcentrifuge and washed twice with PBS 1X. The amastigotes pellet did not contain $>5\%$ trypomastigotes and the supernatant containing trypomastigotes did not have $>5\%$ amastigotes.

Polymerase Chain Reaction Amplification

Polymerase chain reaction (PCR) amplifications were carried out in a volume of 100 µl containing 50 mM KCl; 10 mM Tris-Cl, pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs, 2.5 units Taq polymerase, 100 ng of each oligonucleotide primer, and 1 ng of plasmid DNA as template. The standard amplification conditions were 2 min denaturation 94°C, 1 min annealing at 55°C, and 2 min extension at 72°C, 30 cycles. For amplification of the *Neo*^{r} gene with the oligonucleotide primers Neo1 (AACCATGATTGAACAAGATGGAATTG, nucleotides 1–21 of *Neo*^{r} coding sequence JO1834 and half HincII site) and Neo2 (GCGAATTCAGAAGAACTCGTCAAG, reverse primer of nucleotide 795–778 of *Neo*^{r} coding sequence JO1834, EcoRI site), the annealing temperature was decreased to 42°C, and extension was at 65°C.

Synthesis of ^{32}P-labeled PCR Probes

Molecular probes were generated by PCR amplification, as described previously (15). The ubiquitin probe comprised nucleotides 1–228 (14), *Neo*^{r} gene probe comprised nucleotides 1–795 of the *Neo*^{r} coding sequence (GenBank accession number JO1834) and the *CAT* probe comprised nucleotides 1–660 of the *CAT* coding sequence (GenBank accession number VB0065). Reagent concentrations were the same as the standard reaction, except for those of the nucleotides, which were 50 µCi of α-^{32}P-dCTP or α-^{32}P-TTP (800 Ci/mmol), together with 50 µM of each of the other unlabeled dNTPs.

Plasmid Constructions

All sequences generated by PCR amplification were sequenced to confirm absence of PCR errors.

Construction of pBS:PUB-CAT100. A 667 bp "*PUB*" fragment extending from the ATG codon of the *FUS1* gene, including the intergenic sequences between *FUS1* and *PUB12.5* and terminating four nucleotides upstream of

the *PUB12.5* gene was generated by PCR amplification from a genomic subclone using the oligonucleotides FATG and Ub-CAT1 as primers (18). The PCR product was cloned into the HincII site of pBS:CAT (21) to generate pBS:PUB-CAT. The 729-bp ''100'' fragment comprising the calmodulin small intergenic sequence between the *CALB3* and *CALA5* genes, and including the last 22 nucleotides of the *CALB3* and the first 20 nucleotides of the *CALA5* calmodulin coding sequences (18), was cloned into the blunted BamHI site of pBS:PUB-CAT. The resulting plasmid was pBS:PUB-CAT100.

Construction of pBS:PUB-CATUb100. PBS:PUB-CATUb-100 was generated by cloning the PCR amplified 228 base pair ubiquitin coding sequence (14) between the *CAT* and ''100'' sequences.

Construction of pBS:FN3CU. PBS:FN3CU was generated by inserting the 2.0-kb EcoRV-KpnI fragment containing part of the *PUB12.5* intergenic region, the *CAT* and ubiquitin coding sequences as well as the 100 fragment from pBS:PUB-CAT Ub100 into the EcoRV site of the ''300'' region of PBS: FN3 as shown in Figure 1 (22).

Southern Hybridization

All Southern blots were performed as described earlier (14).

Nuclear Run-on Assays

Nuclear run-on assays were performed essentially as described by Kooter et al. (23). Approximately 2×10^9 log-phase epimastigotes at $1–2 \times 10^7$/mL were harvested for each assay. Nuclei were prepared as described by Srivastava et al. (24). Approximately 1×10^9 nuclei were used in the transcription reaction. Nuclei were incubated on ice for 15 min in 50 mM Tris-Cl, pH 8.0, 2 mM $MnCl_2$, 0.5 mM $MgCl_2$ 25 mM NaCl, 0.5 mM spermidine, 2 mM DTT, 25 U RNase inhibitor, 12.5 mM creatine phosphate, and 70 U creatine phosphokinase. Transcription was initiated by the addition of a nucleotide mix containing 10 mM KCl, 2 mM each of ATP and CTP, 10 μM each GTP and UTP, and 250 μCi each of α-^{32}P GTP and CTP and allowed to proceed at 25°C for 20 min. The reaction was terminated by treatment with DNaseI and proteinase K. The RNA was extracted with phenol and chloroform, precipitated by concentration using 2-butanol and purified by exclusion chromatography using a Sephadex G-50 column. RNA was then used as a probe for a slot blot containing the target DNA, which consisted of 5 μg of base-denatured plasmids baked onto Gene Screen Plus (DuPont-NEN, Boston, MA). Hybridization was at 42°C in 50% formamide, 5X SSC, 5X Denhardt's, 1% SDS and 100 μg/mL carrier tRNA, for 24 h. Following hybridization, the membrane was washed as follows: twice in 2X SSC, 0.1% SDS for 30 min at room temperature; once at 65°C in 0.1X SSC, 0.1% SDS for 20 min; and once in 0.1X SSC, 0.1% SDS for 10 min at room temperature.

Enzyme Assays

*Chloramphenicol acetyl transferase assay (*CAT*). CAT* assays were carried out as described by Bellofatto and Cross (25) with the following changes. Equal numbers of parasites were harvested for each assay. The reaction volume was decreased to 50 μL and incubation time was decreased to 30 min. The reaction products were purified by extraction into 150 μL of xylene and quantitated by scintillation counting following the addition of 3.0 mL of BetaMax (ICN Biomedicals, Costa Mesa, CA). The concentration of protein in the extract was estimated colorimetrically at 595 nm using the Bio-Rad (Hercules, CA) protein assay reagent according to the manufacturer's instructions.

Results

To study the expression of *FUS1* and *PUB12.5* genes, we used two clonal parasite lines containing a replacement of a non-selected reporter gene and a selectable marker. In the previously reported clone TcCL:D6 (26), *CALA2* and *FUS1* were replaced by the *Neo*^{r} (*CALA2*/*Neo*) and *CAT* (*FUS1*/*CAT*) genes. In the present work, the clone TcCL:CAT12.5 was obtained by replacing the coding sequence of *FUS1* with the *Neo*^{r} (*FUS1*/*Neo*) gene sequence and the first ubiquitin unit of *PUB12.5* with the *CAT* coding sequence (*PUB12.5*/*CAT*).

Generation of the T. cruzi *Strain TcCL:CAT12.5*

The tandemly arrayed *FUS1* and *PUB12.5* genes were simultaneously replaced to facilitate a comparative analysis of the two genes and differentiate the transcription of *PUB12.5* from that of other *PUB* genes. In addition, the replacement of *PUB12.5* with a reporter gene would facilitate an estimation of the expression of *PUB12.5* by enzyme assays. Therefore, while *FUS1* was targeted for replacement with the *Neo*^{r} gene, the first ubiquitin unit of the *PUB12.5* gene was simultaneously targeted for replacement with the *CAT* coding sequence. The tandem gene replacement was achieved using the 2.2-kb AflII-DraIII fragment derived from the vector pBS:FN3CU. Recombination was targeted by the ''F'' fragment'' (*CUB* 2.65 to *FUS1* intergenic region) and the ubiquitin coding sequence downstream of the *CAT* gene (Figure 1). The fragment was gel purified and introduced into mid-log *T. cruzi* epimastigotes by electroporation, and a stable G418 resistant population was generated (18). Clonal derivatives of the transformed population were isolated and screened for *CAT* activity. The clones that expressed *CAT* activity were further screened by Southern blot analysis to determine whether

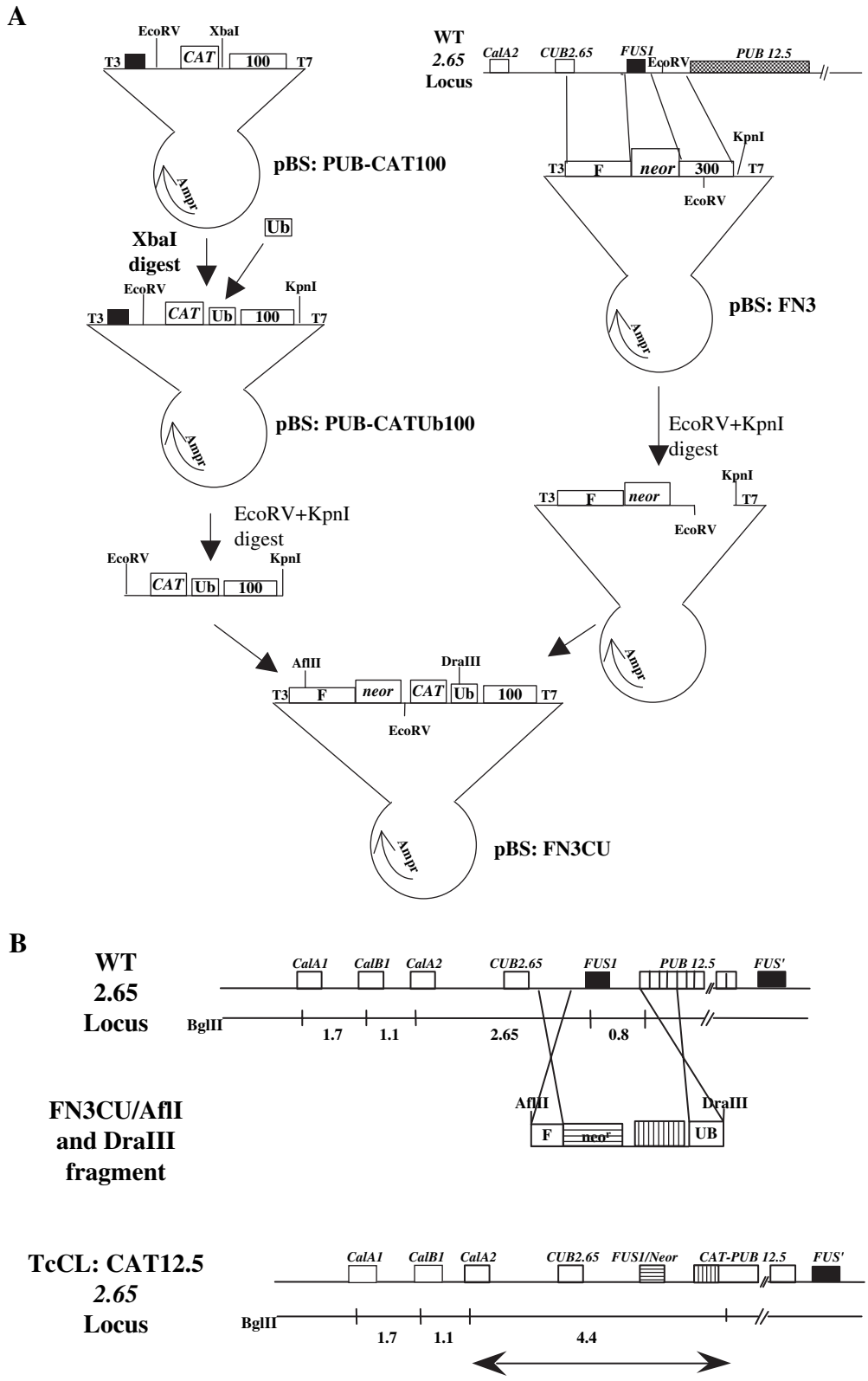


Figure 1. Construction of pBS:FN3CU and proposed mechanism of integration. (A) Details of the pBS:FN3CU plasmid construction from PBS:FN3 (22) are given in the text. Restriction enzyme sites referred to in the text are indicated. T3 and T7 refer to the position of the T3 and T7 primers in BlueScribe (Stratagene) and are shown to indicate the orientation of the insert. (B) The figure shows the proposed events leading to the integration of the FN3CU plasmid into the 2.65 locus and the generation of a truncated *CAT-PUB12.5* fusion gene. Only 9 of the approximately 50-ubiquitin coding sequences (open boxes) of the *PUB12.5* gene are depicted. The antibiotic resistant and the reporter genes were introduced as a result of the replacement of *FUS1* (*FUS*/*Neor*) and some of the ubiquitin-coding units of the *PUB12.5* (*PUB12.5*/*CAT*) gene.

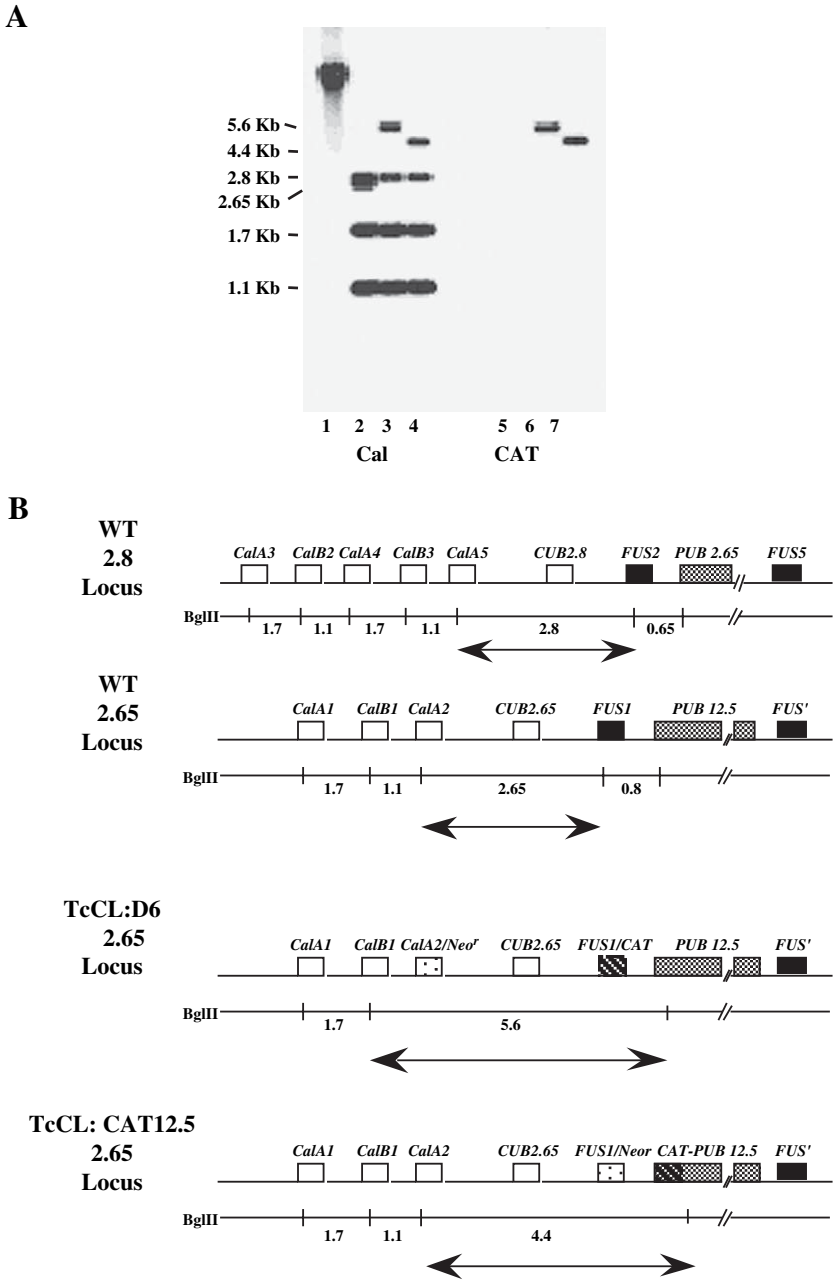


Figure 2. Southern blot analysis of *T. cruzi* strain TcCL:D6 and TcCL:CAT12.5. (A) Replicate blots of uncut (Lane 1) and BglII digested genomic DNA (Lanes 2 to 7) were probed as indicated. Lane 1 contains 3 μg and Lanes 2 and 5 contain 10 μg genomic DNA from wild type (WT) *T. cruzi*. Lanes 3 and 6 contain 10 μg genomic DNA from TcCL:D6. Lanes 4 and 7 contain 10 μg genomic DNA from TcCL:CAT12.5. Abbreviations for the probes are Cal: calmodulin coding sequence and CAT: *CAT* gene. The generation of the probes is described in Materials and Methods. Fragment sizes are kilobases (kb) and were determined based on the mobility of Lambda HindIII, and øX174 HaeIII size standards. (B) The BglII restriction map of the 2.8 and 2.65 calmodulin-ubiquitin loci of WT *T. cruzi* are shown. The arrow highlights the diagnostic 2.8- and 2.65-kb fragments that are detected by the calmodulin coding sequence probe. The arrow underneath the restriction map of the 2.65 locus of TcCL:D6 and TcCL:CAT12.5 highlights the 5.6-kb and 4.4-kb BglII restriction fragment that is diagnostic of the replacements.

the *CAT* gene had integrated at the 5′ end of the *PUB12.5* gene. As depicted in the schematic representation in Figure 1, recombination via the ubiquitin coding sequence could have occurred at any of the ubiquitin coding units in *PUB12.5*. As a result, a truncated *CAT-PUB12.5* fusion gene may have been generated. The Southern blots were used as a screen to ensure that the fusion gene was approximately the same size as *PUB12.5* (data not shown).

The gene replacements in the *2.65* locus of TcCL:D6 and TcCL:CAT12.5 were confirmed by the Southern blot shown in Figure 2A. A comparison of Lanes 2 (WT), 3 (TcCL:D6) and 4 (TcCL:CAT12.5) of the Southern blot showed that the

WT 2.65 kb *Bgl* II fragment, used as a diagnostic of the *2.65* locus (15), is absent in the two stably transformed strains as a result of *Bgl* II restriction sites losses. Instead, new 5.6-kb and 4.4-kb fragments each containing a calmodulin coding sequence are generated. Furthermore, Lanes 6 and 7 show that these fragments are also detected by *CAT* gene probe. This indicates that the *CAT* gene is integrated at the targeted sites in the *2.65* locus of both strains confirming the gene replacements. Figure 2B is a diagrammatic representation of the results.

Transcriptional Analysis

To determine the transcriptional ratios of *FUS1*/*CAT* and *PUB12.5*/*CAT,* we performed a nuclear run-on analysis. Figure 3A shows the results in which the strains TcCL:D6 and TcCL:CAT12.5 epimastigotes were analyzed. The intensity of hybridization to the different coding sequences on the slot blot was quantitated using a PhoshorImager (Molecular Dynamics, Sunnyvale, CA). The table in Figure 3B represents a comparison of the hybridization intensities of *CUB2.65*, *Neo*^{r} and *CAT* genes. The 1:1:1 ratio of the intensities of hybridization of the three coding sequences in the strains indicates that these single copy genes are transcribed at equal levels.

Growth Curve Regulation of Expression of the 2.65 Locus Ubiquitin Genes

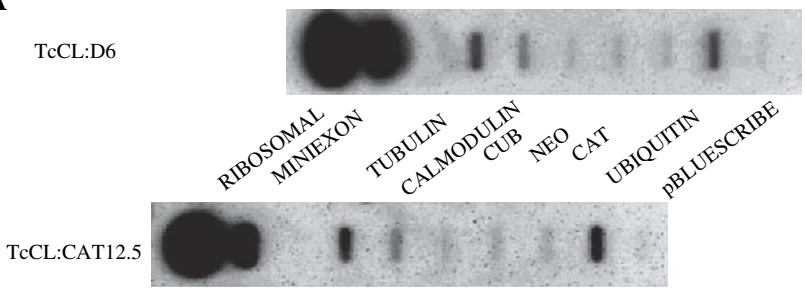
To study the pattern of expression of *FUS1* and *PUB12.5* genes, we analyzed the activity of the CAT gene at varying stages of the growth curve of TcCL:D6 and TcCL:CAT12.5 epimastigotes. The results in Figure 4 indicate that the *FUS1*/*CAT* gene is downregulated 3-fold in stationary phase (90.54 ± 28.74 cpm/μg/min at day 8) with respect to the logarithmic phase (276.5 ± 67.23 cpm/μg/min at day 2), while *PUB12.5*/*CAT* gene expression increases 2-fold during the stationary phase (83.45 ± 28.70 cpm/μg/min at day 8) with respect to the logarithmic phase (39.5 ± 4.10 cpm/μg/min at day 1). The data indicate that while the expression of *FUS1* is downregulated as the parasites approach stationary phase, *PUB12.5* is increased in stationary phase. The comparison of the enzyme activity in logarithmic phase of the two strains showed six to seven times more activity in *FUS1*/*CAT* than in *PUB12.5*/*CAT*.

Developmental Regulation of Expression of the 2.65 Locus Ubiquitin Genes

The expression of *FUS1* and *PUB12.5* was analyzed in epimastigotes, amastigotes and trypomastigotes by determining the activity of the CAT gene in TcCL:D6 and TcCL:CAT12.5. The results of the analysis are reported in Figure 5. The *FUS1*/*CAT* activity in TcCL:D6 increases 1.32 and 2.5 times in amastigotes (428 ± 74.5 cpm/μg/min) and trypomastigotes (837 ± 66.4 cpm/μg/min), respectively, when compared with epimastigotes (324 ± 74.7 cpm/μg/min). Conversely, TcCL:CAT12.5 had 3.3 and 2.3 times less *PUB12.5*/*CAT* activity when epimastigotes (46.2 ± 9.8 cpm/μg/min) differentiate to amastigotes (14 ± 0.4 cpm/μg/min) and trypomastigotes (20 ± 5.2 cpm/μg/min), respectively.

The data indicate that while the expression of *FUS1* increases in trypomastigotes relative to epimastigotes and amastigotes, the expression of the *PUB12.5* gene decreases

A



B

STRAIN	SINGLE COPY GENES	RATIO
TcCL:D6	*Neo*^{r}*;CAT; CUB*	1:1:1
TcCL:CAT12.5	*Neo*^{r}*;CAT; CUB*	1:1:1

Figure 3. Transcriptional analysis of the ubiquitin genes of *T. cruzi*. (A) Nascent RNA synthesized from nuclei of *T. cruzi* strains TcCL:D6 and TcCL:CAT12.5 were used to probe the slot blot. The slot blot was prepared from plasmid clones containing the different trypanosome genes denoted in the blot. The gr2A ribosomal clone is derived from *T. equiperdum* rRNA genes; the miniexon (pABDOS), and p164 tubulin clones are derived from *T. brucei*. Calmodulin, *CUB* and ubiquitin are derived from *T. cruzi* genomic sequences. Neo and CAT refer to pBS:Neo (18), and pBS:CAT (21). pBlueScribe represents the vector control. (B) The intensity of hybridization to the sequences on the slot blot was quantitated using a PhoshorImager. The intensity of *CUB*, *Neo*^{r} and *CAT* genes was compared. The table shows the 1:1:1 ratio obtained from the comparison.

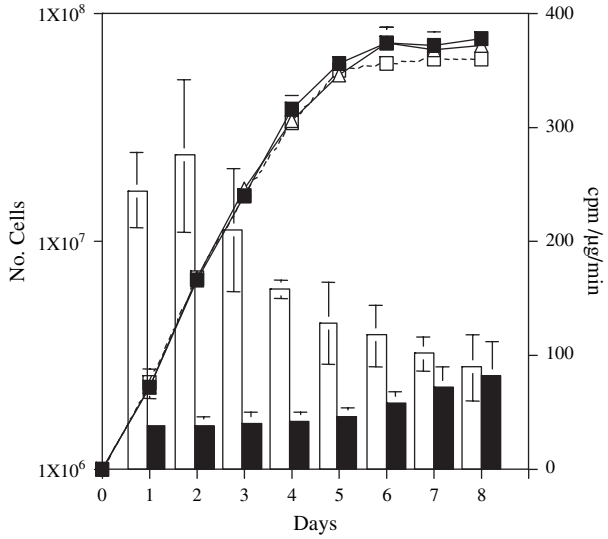


Figure 4. CAT activity of the stable transfected TcCL:D6 (open bars) and TcCL:CAT12.5 (filled bars) strains during different stages of growth. The bar graph shows the amount of specific activity of the enzyme assayed for each day during stages of growth. The line drawing shows the stages of growth of TcCL:WT (■), TcCL:D6 (△) and TcCL:CAT12.5 (□). The x-axis shows the time intervals at which the culture was evaluated and CAT activity tested. The y-axis represents the number of cells and specific CAT activity. Results represent averages of three separate experiments done in duplicate.

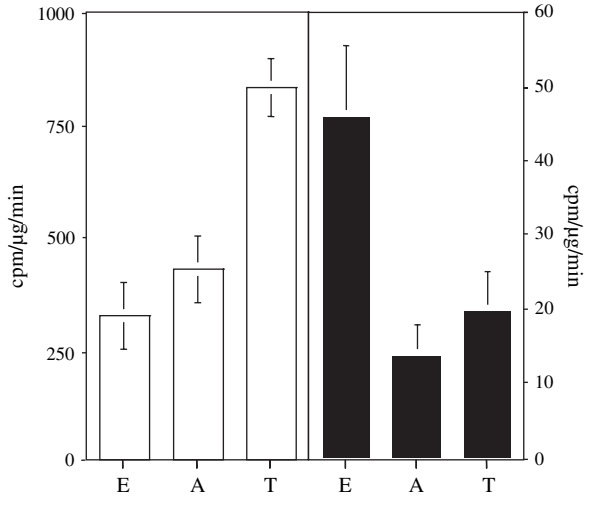


Figure 5. CAT activity of TcCL:D6 (open bars) and TcCL:CAT12.5 (filled bars) clones at different developmental stages. The activity of the enzyme encoded by the CAT reported gene was compared in epimastigotes, amastigotes and trypomastigotes. The columns represent the different developmental forms and the height of the column represents the specific CAT activity. Results represent the average of three separate experiments done in duplicate.

when epimastigotes differentiate into amastigotes and trypomastigotes.

5′-UTRs Sequence Analysis of FUS1 *and* PUB12.5

To determine whether the 5′-UTRs of *FUS1* and *PUB12.5* play a role in the differential expression observed, an alignment of their intergenic sequences was completed (Figure 6). From comparison it is clear that the homology is limited to the region included the two 3′ SASs (from nucleotide –1 to –14) indicating that the two transcripts would also have identical 5′-UTRs. Sequence analysis of the *FUS1*/*neo*^{r} and *PUB12.5*/*CAT* cDNA revealed the native *FUS1* and *PUB12.5 SAS* at position –12 was used during trans-splicing of the recombinant transcript in CnFc7 and TcCL:CAT12.5 (18,21). Consequently, both the *FUS1*/*CAT* and *PUB12.5*/*CAT* have identical 5′ UTR suggesting that this region does not play a significant role in the different levels of CAT expression observed in the two strains.

Discussion

The repetitive nature of most genes in trypanosomes makes it difficult to determine which members of a gene family are expressed. This feature of the parasite gene organization also makes it difficult to assess how expression of different family members might vary with the developmental cycle or growth conditions. The development of transformation techniques, which allows the replacement of genes that are either tandemly linked on the chromosome or present on different chromosomes, has facilitated the analysis of such gene families. This has been particularly true for the analysis of the members of the ubiquitin and calmodulin gene families of *T. cruzi* (21,26).

In this study gene replacements were used to assess the pattern of expression of the tandemly arrayed *FUS1* and *PUB12.5* ubiquitin genes of *T. cruzi*. A comparison of reporter gene activity in logarithmic growing epimastigotes indicated that CAT activity in TcCL:D6 (*FUS1*/*CAT*) was 6- to 7-fold higher than in TcCL:PUB12.5 (*PUB12.5*/*CAT*), indicating that the basal level of expression of these tandemly arrayed genes varied. The analysis was extended to determine how expression of the two genes might vary in response to different stages of growth curve or during the developmental cycle. In the first case, reporter gene activity indicated that while the expression of *FUS1* decreased as parasites reach the stationary phase of growth, expression of *PUB12.5* increased slightly. Expression of the two genes was also shown to vary independently during the developmental cycle. *FUS1* expression remained essentially unchanged in epimastigotes and amastigotes but was increased in trypomastigotes. *PUB12.5* expression, on the other hand, was diminished in amastigotes and trypomastigotes with respect to epimastigotes.

Nuclear run-on analysis showed that in epimastigotes transcription across the *FUS1* and *PUB12.5* genes was constitutive, an observation consistent with the available data on the regulation of gene expression in trypanosomes, suggesting that little or no regulated transcription occurs in these organisms (27).

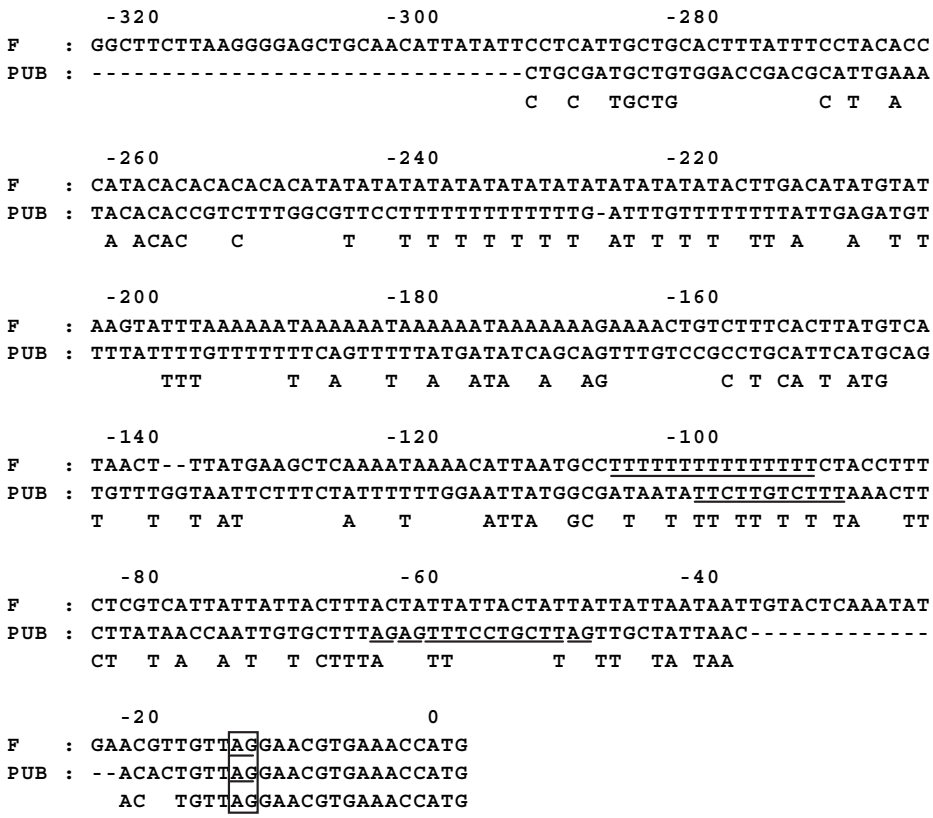


Figure 6. Comparison of the *FUS1* 5′-flanking sequence (F) with *PUB12.5* intergenic sequence (PUB). As indicated, the top line represents the ''F'' intergenic region, the middle line represents the ''PUB'' intergenic region, and the bottom line indicate the similarities between ''F'' and ''PUB'' sequences. The sequences important for trans-splicing as putative polypyrimidine tracts and trans-splicing acceptor site of *FUS1* or *PUB12.5* are underlined. The demonstrated splice acceptor site at –12 for both *FUS1* and *PUB12.5* are underlined and inside a square.

The importance of posttranscriptional mechanisms in the regulation of gene expression in trypanosomes is also emphasized by the fact that regulated transcription initiation has yet to be demonstrated. This is partly due to the fact that polycistronic transcription, coupled with trans-splicing, has made the identification of RNA polymerase II (Pol II) promoters difficult. Therefore, little is known about the sequences that drive the expression of protein-coding genes by Pol II. The only RNA Pol II promoters described so far are for actin (28), hsp70 (29), GARP (30) and SL RNA (31). However, the expression of the reporter gene under the control of the *hsp70* promoter was not increased in response to heat shock, suggesting that the heat shock response in *T. brucei* is not regulated at the level of transcription initiation.

The mechanisms leading to decreased *FUS1* expression as the parasite enters the stationary phase would be interesting to ascertain, because the *PUB12.5* expression increases under the same conditions. As in other eukaryotes (32), the polyubiquitin (PUB) protein of *T. cruzi* apparently is required for the resistance of cells to starvation in stationary phase. On the other hand, *FUS1* is involved in ribosome biogenesis (33) and higher levels of its expression in log phase relative to stationary phase could help support the active translation in growing cells. These results support the earlier observations of Swindle et al. (14), who demonstrated that *PUB* mRNA levels increased in stationary cultures whereas those of the *FUS* genes were greatly diminished.

Trypanosomatids appear to lack polymerase II promoter regulation. Consequently, they control their mRNA steady-state levels mainly throughout posttranscriptional level and much of this control is exerted by the gene UTRs, trans-splicing efficiency and translation (34,35). Because *FUS1* and *PUB12.5* transcripts have identical 5′ UTRs, it is suggested that these sequences cannot play roles in either differential mRNA stability or translatability. However, functional analysis will be necessary to evaluate whether the 5′ and 3′ UTRs and/or a trans-splicing efficiency mechanism of *FUS1* and *PUB12.5* have a significant role in determining the different levels of activity observed.

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