Variation of transient gene expression within single lineages of *Trypanosoma cruzi*

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SUMMARY

The biodiversity of *Trypanosoma cruzi* is one of the main factors complicating the understanding of its molecular epidemiology. As an alternative to classical genetic methods, investigators have used DNA-mediated transformation techniques to study this diversity. Recently, transient expression data were shown to correlate with the genetic data. This led investigators to speculate on a potential speciation within clonal populations of *Trypanosoma cruzi*. To further test the phylogenetic significance of transient expression analysis in *Trypanosoma cruzi*, multiple plasmids were used to drive the expression of a reporter gene in different parasite populations. In our study, population specific expression of the reporter gene was observed but the variability revealed by these transient expression assays was very large and did not follow the grouping of *Trypanosoma cruzi* populations in 2 lineages. In this report, we discuss some of the limitations of transient assays on such a diverse parasite.

Key words: *Trypanosoma cruzi*, phylogeny, transfection, chloramphenicol acetyl transferase gene, calmodulin–ubiquitin associated gene (CUB), Chagas' disease.

INTRODUCTION

The ability to introduce exogeneous DNA into parasitic protozoa has furthered studies on gene expression and regulation (Ajioka & Swindle, 1993; Beverley & Turco, 1995; Swindle *et al*. 1995), promoter characterization (Crabbs & Cowman, 1996), and parasitic infectivity (Pham, Qi & Gottesdiener, 1996; Garg, Nunes & Tarleton, 1997). In *Trypanosoma cruzi*, transient gene expression driven by the ribosomal RNA promoter has also been shown to corroborate population genetic analysis which group the species into 2 major lineages (Tibayrenc *et al*. 1986; Tibayrenc, 1995; Tyler-Cross *et al*. 1995; Souto *et al*. 1996; Nunes, de Carvalho & Buck, 1997). This division into 2 lineages may not have medical or epidemiological relevance since, first, lineage 1 has been shown to be heterogeneous and different phylogenetic sub-groupings have been described (Brisse, Barnabé & Tibayrenc, 1999); second, each lineage is made of a number of biologically distinct clonal populations or clonets (Sanchez *et al*. 1990; Laurent *et al*. 1997); third, epidemiologic data have divided *Trypanosoma cruzi* in at least 3 polar groups (Miles *et al*. 1978; Tibayrenc & Breniere, 1988).

In the present report, the working hypothesis is that phylogenetic divergence correlates with the variability in molecular biology of the parasite. For this purpose, we used transient gene expression to compare phylogenetically different stocks of *Trypanosoma cruzi* belonging to major clonal populations within the 2 major lineages. Some stocks exhibited high levels of reporter gene expression while others expressed none. These differences did not correlate with the phylogenetic data.

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MATERIALS AND METHODS

Parasite stocks

The stocks were selected according to previous characterization (Tibayrenc *et al*. 1986, 1995) in order to represent 3 different groups of clonal genotype (clonet) within the 2 lineages. Clonets 39 and 43 are closely related to each other and belong to lineage 1, while clonet 20 is distantly related to either 39 or 43 and belongs to lineage 2. Geographical origins and phenotypic characteristics are presented in Table 1. All stocks have been cloned by micro-

Table 1. Stocks of *Trypanosoma cruzi*

Stock	Geographical origin	Clonet*	Lineage†	Doubling time‡
SO3	Bolivia	39	1	78
NR	Chile	39	1	51
Esquilo	Brazil	20	2	50
CL	Brazil	43	1	20

* Clonet was determined by characterization of at least 4 isozymic loci (Tibayrenc *et al*. 1986).
† Lineage was given according to Tibayrenc *et al*. (1995).
‡ Doubling times for all stocks but CL were determined previously (Laurent *et al*. 1997).

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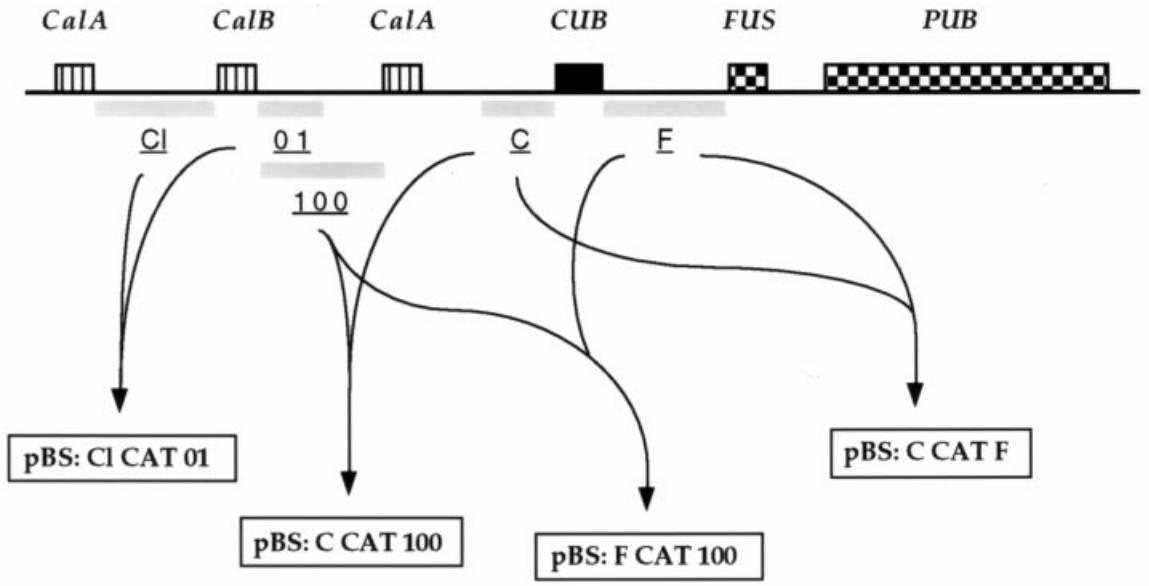

Fig. 1. Construction of the plasmids. *Cal*, calmodulin gene; *CUB*, calmodulin–ubiquitin associated gene; *FUS*, ubiquitin–fusion gene; *PUB*, polyubiquitin genes. The grey boxes indicated the region of the genome used to flank the reporter gene.

manipulation with verification under the microscope. All were maintained as epimastigotes in Liver Infusion Tryptone (LIT) medium (Camargo, 1964) supplemented with 10 % fetal calf serum (Complete LIT Medium) at 28 °C. Cells in logarithmic phase were used in all experiments.

Nucleic acid hybridization analysis

Cells were disrupted in the presence of 1 % sodium dodecyl sulphate and total cellular DNA was isolated by ethanol precipitation in the presence of 1·6 M potassium acetate. For slot blot analysis, different amounts of DNA were loaded on the membrane after alkaline denaturation.

Synthesis of ^{32}P-labelled probes was done by polymerase chain reaction (PCR) as previously described (Gillespie *et al*. 1993). Hybridizations for Southern blots and slot blots were carried out as previously described (Hariharan, Ajioka & Swindle, 1993). The genome content of 2 stocks, namely SO3 and CL, was found to be approximately equal (Laurent & Swindle, unpublished data).

Transient expression assays

We used the chloramphenicol acetyl transferase (CAT) gene as reporter gene and the plasmid BlueScribe (+) as the DNA vector (Stratagene, USA).

The parasites were harvested by centrifugation and 3×10^8 cells were incubated on ice with 100 μg of plasmid for 10 min. Cells were transfected in a 2 mm electrode gap cuvette, by an electric pulse of 350 V, 1500 μF, 24 Ω using an ECM® 600 (Genetronics, Inc., USA). Cells were then resuspended in 10 ml of complete LIT medium and incubated at 28 °C. At each time-point, $1{\cdot}5 \times 10^8$ parasites were harvested and washed 3 times in TEN buffer (Tris 40 mM, pH 8, EDTA 1 mM, pH 8, NaCl 15 mM). The cell pellet was resuspended in 100 μl of Tris 0·25 M, pH 8, chilled on ice. Cells were then broken open by 3 cycles of freezing and thawing and the suspension was incubated at 65 °C for 10 min. After centrifugation, the supernatant was assayed for chloramphenicol acetyl transferase activity by incubation for 1 h at 37 °C in the presence of butyryl-coenzyme A and ^{14}Cchloramphenicol. The acetylated chloramphenicol was extracted in xylene and the counts were read in a LS 5000CE Scintillation Counter (Beckman Coulter, Inc., USA) after mixing with 2 ml of BetaMax ES (ICN Biomedicals, USA).

Plasmid construction

The subscript in the plasmid name indicates the origin of the template DNA: S for SO3 and C for CL. In Fig. 1, the sequences used in the construction of each plasmid and controlling the expression of the reporter gene are shown (Ajioka & Swindle, 1993; Gillespie *et al*. 1993; Hariharan *et al*. 1993).

RESULTS

To test our hypothesis, we chose 4 stocks belonging to 3 different clonal populations (clonet 20, 39 and 43) which appear to be widespread and very frequent in South America and were called major clones (Tibayrenc & Brénière, 1988). The following considerations helped us select specific clonets that were

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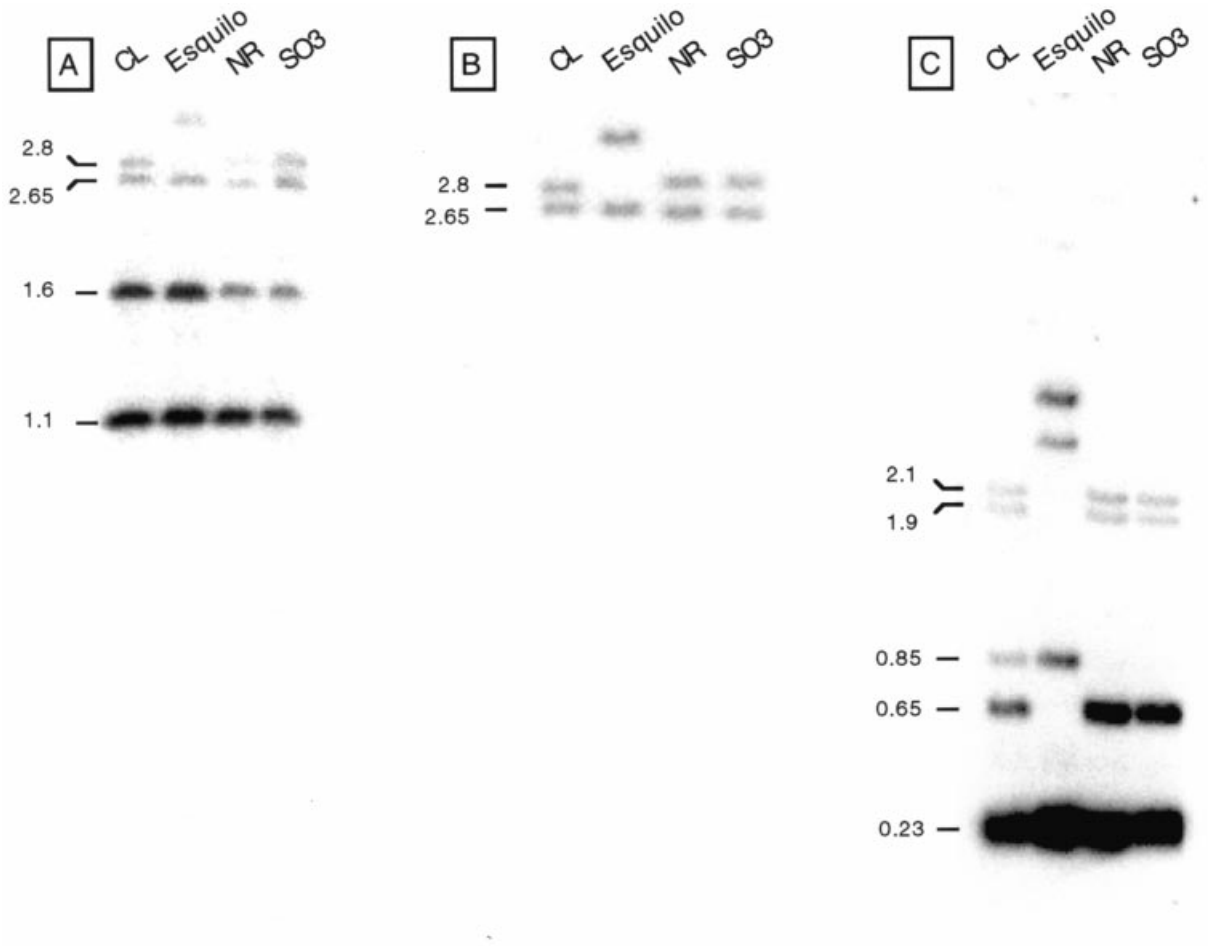

Fig. 2. Southern blot analysis. Each lane contains 2 μg of genomic DNA digested with *Bg*lII. Hybridization is done in (A) with a calmodulin-derived probe, in (B) with a *CUB*-derived probe and in (C) with an ubiquitin-derived probe. The molecular weights of CL restriction fragments are given on the side of each panel.

shown to have different biological characteristics (Sanchez *et al*. 1990; Laurent *et al*. 1997). Stocks from the clonet 20 were shown to be statistically more infectious, faster growing *in vitro*, and more capable of producing metacyclic trypomastigotes *in vitro* than were the stocks of the clonet 39. Lastly, although the biological properties of the clonet 43 were not comparatively so well known, it is part of the lineage 1 and phylogenetically very closely related to the clonet 39. The stock CL, chosen as the reference strain for the *Trypanosoma cruzi* genome project, belongs to the clonet 43.

Each expression plasmid was constructed by flanking the chloramphenicol acetyl transferase gene with sequences originating from the calmodulin–ubiquitin associated gene (CUB) loci (Swindle *et al*. 1988) of *T. cruzi* (Fig. 1). Southern blot analysis was used to reveal the structure of the 2 CUB loci for the 4 stocks studied. The pattern of hybridization to *Bg*lII restricted genomic DNA, using 3 different probes, is presented in Fig. 2. The CUB loci for each stock include the calmodulin genes followed by a single CUB gene which is followed by ubiquitin genes. Since the basic organization of the loci was conserved, 5 plasmids derived from these loci were used to compare transient expression in the different stocks.

In the first experiment, chloramphenicol acetyl transferase assays were performed following electroporation of 3 CL-derived expression plasmids into mid-logarithmic phase epimastigotes of each stock (Fig. 3A). Independent of the plasmid used, CL expressed a high level of chloramphenicol acetyl transferase while a very low level of chloramphenicol acetyl transferase activity was observed for NR, Esquilo and SO3. Esquilo expressed 80 times less chloramphenicol acetyl transferase activity than CL when transfected with pBS:F_CCAT100$_C$, SO3 and NR expressed 16 and 24 times less chloramphenicol acetyl transferase activity than CL when transfected with pBS:Cl_CCAT01$_C$. Since these plasmids carried DNA sequences derived only from the stock CL, this experiment could suggest a strict stock specificity to expression. To address this question, a fourth plasmid containing flanking regions derived from SO3 DNA was constructed. This plasmid, pBS:C_SCATF$_S$, and the corresponding CL-derived plasmid pBS:C_CCATF$_C$ were electroporated into

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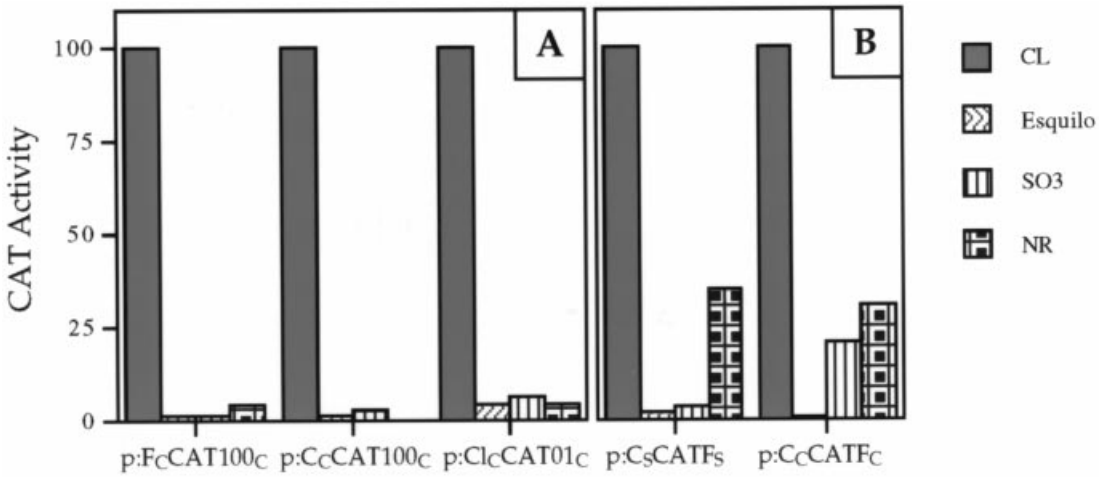

Fig. 3. Graphic presentation of chloramphenicol acetyl transferase activities of the 4 stocks electroporated with the 5 different plasmids. Activities were given as a percentage of CL-specific activity. In (A), plasmids contain regulatory elements from CL. In (B), regulatory elements of the *CUB* gene from CL and from SO3 are compared.

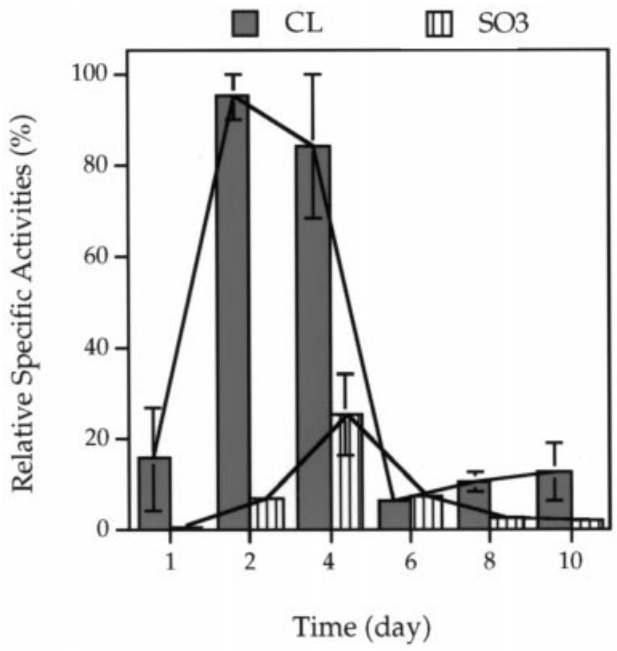

Fig. 4. Relative chloramphenicol acetyl transferase activity during SO3 and CL *in vitro* growth. Chloramphenicol acetyl transferase was assayed for each time-point on the same number of cells. CL highest specific activity, during the course of the experiment, has been taken as 100% and other specific activities were expressed as a percentage.

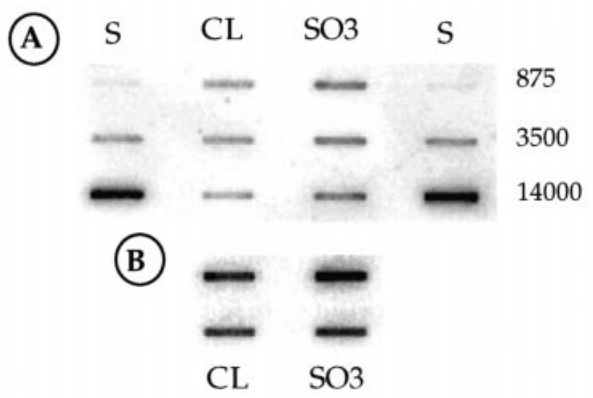

Fig. 5. Slot blot analysis. In (A), 50 μg of genomic DNA by slot were loaded. In lane S, 32 ng, 8 ng and 2 ng of the plasmid p:C_sCATF_s were loaded by slot. Numbers on the side indicate how many copies of reporter genes per genome the density of hybridization corresponds to. In (B), 200 μg of genomic DNA were probed with calmodulin-derived sequence and the average intensity of hybridization was taken as internal control.

the 4 stocks and chloramphenicol acetyl transferase activities compared (Fig. 3B). If the transient gene expression was stock specific, higher expression would be expected in the homologous cells. On the contrary, when transfected with pBS:C_sCATF_s, SO3 produced 30 times less activity than CL. These comparisons showed that chloramphenicol acetyl transferase expression was not influenced by differences in the primary structure of the regulatory sequences and it was not dependent on the origin of these sequences, suggesting that another factor was responsible for the differences of activity between stocks. Additionally, experiments comparing chloramphenicol acetyl transferase activities after a complete cell cycle showed no correlation between transient chloramphenicol acetyl transferase expression and growth rate.

Parasites undergo many cellular changes during their growth which could affect the level of gene expression. Consequently, we investigated the variation in chloramphenicol acetyl transferase expression during the *in vitro* growth of SO3 (the slowest grower) and CL (the quickest grower). After transfecting these 2 stocks with pBS:C_sCATF_s, chlor-

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Table 2. Chloramphenicol acetyl transferase (CAT) expression in transfected epimastigotes (CL relative activity was normalized on SO3 activity.)

Stocks	Number of CAT genes per genome	Relative CAT activity
CL	700	11
SO3	900	1

amphenicol acetyl transferase activity was measured every 2 days. The chloramphenicol acetyl transferase activity in CL was consistently higher than in SO3 (Fig. 4). However, several differences in their pattern of expression suggested that while the plasmid was transfected and expressed, the kinetics of expression differed between the 2 stocks. First, the peak of chloramphenicol acetyl transferase activity occurred earlier in CL (between 2 and 4 days) than in SO3 (4 days) and the CL peak was on average 4 times higher than the SO3 peak; second, the increase of activity from day 1 to the peak was much higher for SO3 (34 times) than for CL (6 times).

To determine whether low chloramphenicol acetyl transferase activity into SO3 was due to inefficient electroporation, pBS:C_SCATF_S was electroporated into SO3 and CL and the chloramphenicol acetyl transferase activity and the plasmid copy number were analysed. Although CL expressed substantially more chloramphenicol acetyl transferase activity than SO3, both strains carried the same number of chloramphenicol acetyl transferase genes per parasite (Fig. 5 and Table 2) suggesting an equally efficient transfection. Both stocks also appear to be able to maintain the plasmid for extended periods since chloramphenicol acetyl transferase activity could be detected up to 16 days post-transfection.

DISCUSSION

NR and SO3 are both from the clonal population or clonet 39. The clonal structure of *T. cruzi* populations (or clonets) has been shown to have a major impact on their biology. In these early studies, stocks of clonet 39 were shown to have important biological properties significantly different to the stocks of the distantly related clonet 20 (Sanchez *et al.* 1990; Laurent *et al.* 1997). CL (clonet 43), although not so well characterized, is very closely related to NR and SO3. The 3 clonets (20, 39 and 43) also represent both lineages. Because of the clonal structure of *T. cruzi* populations, one could envisage shared mechanisms for gene expression between closely related stocks while more distantly related populations would exhibit lineage-specific expression. But the question remains of the medical and biological relevance of the different lineages. NR, SO3 and CL belong to the lineage 1 and were compared to Esquilo from clonet 20 and lineage 2. Previous studies demonstrating lineage-specific RNA polymerase I gene expression support such a hypothesis (Tyler-Cross *et al.* 1995; Nunes *et al.* 1997). These studies, however, focused only on slow-evolving sequences (Hori & Osawa, 1986; Barta, 1997) and their correlation to the lineage division could have been predicted since the 2 lineages have been shown to have diverged very early in *T. cruzi* evolution (Tibayrenc, 1995).

In the current study we analysed the expression of protein-coding genes between NR, SO3, CL and Esquilo. Genomic Southern analysis of the calmodulin–ubiquitin loci used as the source of regulatory sequences in this analysis correlated with the evolutive divergence between the stocks. NR and SO3 (clonet 39, lineage 1) were indistinguishable and differed only slightly from CL (clonet 43, lineage 1) while Esquilo (clonet 20, lineage 2) exhibited the most divergent pattern.

In contrast to the genomic Southern analysis, transient gene expression did not correlate with the phylogeny of the parasite stocks tested. In all cases, CL expressed chloramphenicol acetyl transferase more efficiently than the other stocks. This enhanced expression was independent of the source of the flanking sequences since SO3-homologous plasmids were also most highly expressed in CL. The temporal pattern of chloramphenicol acetyl transferase expression did, however, differ between CL and SO3. The peak of expression was earlier for CL than it was for SO3. This result demonstrates the importance of optimizing transient expression for different stocks. Had we terminated the analysis after 1 or 2 days, as is normally the case, we would have concluded that SO3 was a non-expressor rather than a delayed expressor. We also would have concluded that there was a strict stock specificity to transient gene expression which would have been a significant overstatement. The differences between SO3 and CL which are phylogenetically closely related (clonet 39 and 43 respectively) go further than a delay in expression: they both contain high chloramphenicol acetyl transferase gene copy number during the course of the kinetics. However CL had a peak of activity after more than 2 cell cycles by an increase of activity from day 1 of 6-fold, when SO3 after less than 1 cell cycle increased its chloramphenicol acetyl transferase activity from day 1 by 34-fold. It is impossible with our data to understand the basis of these biological differences between the 2 stocks, but they are important enough to doubt the suitability of transient expression data to assess phylogenetic relationships between the major medically relevant populations of *T. cruzi*. The capacity to express chloramphenicol acetyl transferase is independent of the primary structure of the regulatory sequences since it is independent of the expression plasmid used. This raises the possibility that differences exist

between *trans*-acting factors regulating gene expression between populations. The study of such differences may allow us to understand the complex evolutive pattern of the *T. cruzi* species and indirectly its epidemiology. Also the fact that different *T. cruzi* stocks exhibited different capacities to express exogeneously introduced genes could have profound consequences for experiments using transformation to identify genes which are able to confer different phenotypes on diverse stocks. For example, the ability to transfer a virulent phenotype from a virulent to a non-virulent parasite stock would be compromised if the recipient parasite stock is unable to efficiently express transfected genes. For this purpose, since it is technically hard to isolate mutant strains in *T. cruzi*, using stocks from the same clonet may greatly improve the chance to obtain positive results.

Based on the present study, the pattern of reporter gene expression between the 4 stocks tested did not follow any obvious phylogenetic divisions. NR and SO3 (clonet 39 and lineage 1) resembled Esquilo (clonet 20 and lineage 2) more than they resembled CL (clonet 43 and lineage 1). The degree of biodiversity of *T. cruzi* is very high, and for decades the number of relevant phylogenetic subdivisions has been investigated and debated. Recent genetic analyses have indicated that lineage 1 is substantially more diverse than lineage 2 (Brisse *et al*. 1999). This apparent heterogeneity may be reflected in the present study. However, the capacity to express plasmid-borne genes is likely to be synapomorphic to clonet 43. The observation is supported by preliminary analysis showing higher average chloramphenicol acetyl transferase expression in several stocks from clonet 43. Additional studies using many more stocks will, however, be needed to confirm this result. But if it is true, this is another reason why transient expression data might not be suitable in assessing phylogenetic relationships among *T. cruzi* populations.

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