Minimum free energy predicted base pairing in the 39 nt spliced leader and 5’ UTR of calmodulin mRNA from Trypanosoma cruzi: influence of the multiple trans-splicing sites

FRANKLYN SAMUDIO1,2 and ADEILTON BRANDÃO1

1Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz, Fiocruz, Av. Brasil, 4365, 21040-360 Rio de Janeiro, RJ, Brazil
2Instituto Conmemorativo Gorgas de Estudios de la Salud, Ave. Justo Arosemena, Entre calle 35 y 36, Apartado Postal 0816-02593, Panamá, República de Panamá

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ABSTRACT

We analyzed the compositional changes and the stable base pairs in the predicted secondary structure of the 5’ UTR calmodulin mRNA in T. cruzi. The three copies of calmodulin in T. cruzi genome display variable position of the trans splicing sites and give rise to several mRNA that differs slightly on 5’ UTR composition in the epimastigote stage. We show that the pattern of high probability base pairs in the minimum free energy predicted secondary structures of the calmodulin 5’ UTR remains unchanged despite the nucleotide composition variation. However, the 39 nt spliced leader (mini-exon, the 5’ exon sequence transferred to trypanosome mRNAs by the mechanism of trans splicing) shows a variable pattern of high and low probability base pairing as consequence of the altered composition of the 5’ UTR.

Key words: trans-splicing, untranslated region, calmodulin, epimastigote, RNA secondary structure, minimum free energy.

INTRODUCTION

Though T. cruzi is typically a eukaryote, it displays unusual features like polycistronic transcription, absence of introns in most of the genes, and processing of protein-encoding RNA by the mechanism of trans-splicing coupled to polyadenylation (Araújo and Teixeira 2011). As parasite, T. cruzi accomplishes its life cycle by moving from invertebrate to vertebrate hosts (Coura 2014). This transition submits the parasite to physical and chemical shifts that include wide variations in temperature, nutrient availability, osmotic and environmental pressure. Trypanosome transcriptome analysis shows that the processing of the primary polycistronic transcript from tandem arrayed genes might generate several variants of the corresponding monocistronic mRNA, mainly due to the use of additional sites of trans-splicing and polyadenylation (Nilsson et al. 2010). These facts call attention to the problem of the alterations in the 5’ UTR secondary structure in certain genes that might be caused by changes in length and composition of 5’ UTR derived from distinct
trans splicing sites. To shed light on this problem, we analyzed in this work the effect on both the 5’ UTR and the 39 nt SL (mini-exon, the 5’ exon sequence transferred to trypanosome mRNAs by the mechanism of trans splicing) of the multiple trans splicing sites of the calmodulin gene in the epimastigote stage of *T. cruzi* Y strain.

**MATERIALS AND METHODS**

*T. cruzi* Y strain epimastigotes were cultivated at 28 °C in brain heart infusion (BHI) (Becton, Dickinson and Company) supplemented with 10% of heat-inactivated fetal bovine serum (Life technologies). At least 2x 10⁸ mid-log phase epimastigotes were recovered by centrifugation at 3,000 rpm for 10 min and washed three times with PBS. The RNA was extracted by Trizol (Life technologies) according to manufacturer instructions, and resuspended in 30 µL of water. Calmodulin 5’ UTR was obtained by RT-PCR using primers targeted to the spliced leader (forward primer based on the first 25 bases of the 39 nt spliced leader: 5’ AACTAACGCTATTATTGATACAGTT 3’) and calmodulin 3’ UTR common to all copies (reverse primer: 5’ GTCACTGTCGGCTTCGCT 3’). Two micrograms of RNA were used to produce cdNA in a standard reaction at 50 °C for 90 min with 2 U of Super Script III (Life technologies) and 500 nM of the anchored primers T(13)A, T(13)G, T(13)C. Then a PCR was carried out using Platinum® Taq DNA Polymerase High Fidelity (Life technologies) and 100 nM of primers forward and reverse at 94°C for 2 min followed by 35 cycles of 97 °C for 15 s, 60 °C for 20 s, 72 °C for 30 s and a final extension of 72 °C for 7 min. Pyrosequencing were carried out at the high-throughput sequencing facility of the Instituto Oswaldo Cruz – Fiocruz. The sff file (Roche 454 GS Junior output) was converted to a fastQ file by the sff2fastq and further edited by program CANGS (Pandey et al. 2010). The sequences have been submitted to Sequence Read Archive (SRA) of Genbank-NCBI under accession codes: Experiment: SRX734426, Run: SRR1614234. Two hundred bases upstream of the start codon from each copy of the calmodulin locus from CL Brener Esmeraldo haplotype type were used to generate sequence alignments through Clustal Omega in the UGENE package. Though the CL Brener clone and Y strain have been allocated to different *T. cruzi* DTUs (DTU VI for CL Brener and DUT II for Y strain) (Zingales et al. 2009), the tandem arrangement of the calmodulin gene is invariable in *T. cruzi* strains belonging to different DTU (Brandão and Fernandes 2006). Complete 5’ UTR sequences (including the 39 nt SL) were submitted to the RNAfold in the viennaRNA Web Services under default parameters for the prediction of the base pair probabilities in the minimum free energy structure (Gruber et al. 2008).

**RESULTS AND DISCUSSION**

The calmodulin locus is organized as three tandem copies with 450 nt ORF (copy accession numbers: TcCLB.507483.50, TcCLB.507483.39, and TcCLB.507483.30). Five point mutations occur among the three ORF, but only one results in amino acid change. The 5’ UTR in TcCLB.507483.50 is distinct from the two other copies but it is almost identical in copies TcCLB.507483.39 and TcCLB.507483.30. We have used the point mutation in nucleotide position 69 of the ORF to distinguish the origin of each mRNA in the last two copies. Figure 1 shows a diagram of the calmodulin locus in the *T. cruzi* CL Brener haplotype Esmeraldo. Two spacers of 1,388 and 702 nt separate the pairs TcCLB.507483.50 – TcCLB.507483.39 and TcCLB.507483.39 – TcCLB.507483.30, respectively.

The observed trans splicing sites in each copy were as follows (in bold):

a) TcCLB.507483.50: 4;

b) TcCLB.507483.39: 8;

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As expected, most of these sites are defined by the canonical dinucleotide AG, but non-canonical trans-splicing sites such as CA, CC, CG, GG and TG were observed as well. The processing of polycistronic transcripts in trypanosomatids might result in abundance variation of the final mRNA of a particular gene or group of genes (Clayton 2014). According to transcriptome data provided by Li et al. (2016), the calmodulin expression level in epimastigote stage varies among its copies, indicated here are by average log-transformed quantile-normalized cpm expression values (in bold):

a) TcCLB.507483.50: 7.79,
b) TcCLB.507483.39: 8.17,
c) TcCLB.507483.30: 8.67

These expression values are not linearly related to the number of trans-splicing sites for each copy, although the copy with the lowest expression level (TcCLB.507483.50) is also the one with fewer trans-splicing sites. This copy is located in one of the extremities of the calmodulin locus (see fig. 1).

A given RNA molecule can be viewed as a thermodynamic ensemble of structures and does not necessarily exhibit a unique secondary structure under cell physiological conditions (Ding and Lawrence 2003). Thus, instead of looking at the predicted secondary structure, it is more informative to look at the paired base segments that occur more frequently in the thermodynamical ensemble of low free energy structures. These high probability paired base segments are certainly to be present in the actual secondary structure for that RNA (Mathews et al. 2010). The multiple trans-splicing sites shown to be present in calmodulin locus give rise to a wide range of length variation in the final mRNA, implicating in millions of alternative secondary structures for the 5' UTR. We asked whether the variable 5' UTR length is directly related to the degree of structured sequence in each mRNA from the three gene copies. To address this question, we used a reliability measure of the RNAfold software: colorized base pairing probabilities for each 5' UTR. As shown in fig. 3, the 39 nt spliced leader (shown partially in black line rectangle) may assume different configurations with a variable number of base pairs. The most stable ones (high probability segments in red) occur in the 5' UTR of intermediate length (104 – 150 nt). The paired bases (stems) of the spliced leader are closer to beginning of the 5' UTR. However, most of the paired bases are of low probability. It is noteworthy how the predicted mfe structure of 39 nt SL changes with the composition of the 5' UTR, which is a consequence of the ”movable” trans-splicing site. As shown in figure 2, the mfe predicted secondary structure of the SL sequence alone (39 nt) displays
In the mid 1990s, researchers have shown that SL from *Leptomonas collosoma* and *T. brucei* displayed two main structures (LeCuyer and Crothers 1993). Similar work *in vivo* (permeabilized cells) demonstrated that the SL alternates between these structures with predominance of structure 1 (Harris et al. 1995). Although some of the predicted SL structures in the 5' UTR analyzed here roughly approached the structures characterized earlier, these results should be interpreted with caution because the computer prediction of RNA structures does not exclude the possibility that less thermodynamically stable structure are the real biological (*in vivo*) structures. What the prediction allows us to ascertain is that only the first 5-8 bases of SL remain single base at high probability for most of the 5' UTR sequences in calmodulin mRNA. Considering that millions of structures might exist beyond the most thermodynamically stable (mfe structure), it cannot be ruled out that structures not selected by the current algorithms are completely absent of any functional activity *in vivo*.

According to mfe predictions presented here, the thermodynamically stable segments in the 5' UTR of calmodulin mRNA do not change the amount of paired bases with the additional trans splicing sites used to process the primary mRNA. It is also worth noting that the different length and composition of the 5' UTR do not affect the formation of a stem loop in the high probability 41 nt segment close to the calmodulin start codon (black line circle in fig. 3).

With the exception of the stem loop close to start codon, the pairing probability for *T. cruzi* calmodulin 5' UTR sequences in the epimastigote stage shows that most of these segments remain single base, *i.e.*, do not form a secondary structure. For the 5' UTR this is not a surprise, as the 5' leader sequence in the mRNA is expected to be lightly structured, unless a large portion of the 5' UTR is involved in gene expression control and modulation of translation rates (Hinnebusch et al. 2016).

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**Figure 2** - Predicted mfe secondary structure with base pairing probabilities for the 39 nt spliced leader present in mRNA from *T. cruzi*. The sequence was folded by RNAfold under default parameters.

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a single stem loop with 5 bp helix. In this predicted structure, the highest probability is assigned to the unpaired bases at the 5' end (the 12 single bases in red). This structure is highly improbable *in vivo* because the 39 nt SL does not exist isolated in the cell: either it is coupled to original donor RNA or to the 5' end of messenger RNA. By comparing this same 39 nt SL segment coupled to the variable 5' UTR segments, another picture emerges from the predicted structures: the single stem loop disappears to give rise to a variable number of low and high probability short helices (paired bases).

In all 5' UTR analyzed here, only the first 5 - 10 nucleotides at 5' end of SL appears unpaired at high probabilities. Most of the remaining bases form either low or high probability base pairing with 5' segments of the various 5' UTR. In this specific case, the 39 nt spliced leader adapts its secondary structure to the composition of the 5' UTR. This implies an importance to composition alterations in the 5' UTR from *T. cruzi*, in particular those caused by changes in the trans splicing site.
Figure 3 - Predicted mfe structure with base pairing probabilities for the calmodulin 5' UTR in *T. cruzi* epimastigote. The 5' UTR sequences (grouped by copy origin) were joined to 39 nt SL (black line rectangle showing partially paired segment) and folded by RNAfold. Colors in the schematic structure refer to probability of base pairing or unpairing (remaining as single base). Red segments contain the paired (or unpaired) bases of the highest probability. Inside the black line rectangle are the paired bases that the 39 nt spliced leader forms with either itself or the 5' UTR sequence. Most of these SL paired bases are of low probability. Black line circles indicate the stem loop with high probability paired bases near the start codon of the calmodulin mRNA. Numbers below the major single base segment denote the 5' UTR length (plus the spliced leader).
CONCLUSIONS

Despite alterations in nucleotide composition and mRNA expression level for the three gene copies, the calmodulin 5’ UTR tends to maintain roughly the same pattern of stable paired/unpaired bases. In contrast, the 39 nt spliced leader sequence shows a variable segment of high and low probability paired bases.

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