15

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USEFULNESS OF A RECURSIVE PCR TO INSERT A HIGH AFFINITY APTAMER INTO THE CALMODULIN MINOR SPACER of *Trypanosoma cruzi*.

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Abstract

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease, an endemic illness in Panama. In this parasite, regulation of gene expression is mainly a post transcriptional process. Has been suggested that RNA-binding proteins have a key role in this gene regulation. Calmodulin is a highly conserved calcium sensor protein, encodes by three genes separated by two intergenic spacers (major and minor spacer). The study of molecular basis that led RNA-proteins interactions in *T. cruzi* could contribute to understand the biology of the parasite to develop novel strategies for disease control. We developed a methodology based on a recursive PCR to insert a streptavidin high affinity aptamer into the sequence of the minor spacer, as the basis for the development of a protein capture system. The complete calmodulin locus sequence was download from GenBank (AAHK01001263.1). Previous reported aptameric sequence (83bp) was manipulated for primer design. Sequences were edited with UGENE bioinformatic software. Calmodulin minor spacer (CMS) sequence was fragmented into two regions. Primers were designed to flank the two regions, the inner forward/reverse oligos attached to half aptamer sequences each. Final recursive PCR was able to amplify the Calmodulin minor spacer incorporating the aptamer. Sequence

was confirmed by Sanger sequencing. Recursive PCR represent a useful tool to insert high affinity sequences as aptamers to study specific protein-RNA interactions.

Keywords
Trypanosoma cuzi, Aptamer, recursive PCR.

UTILIDAD DE LA PCR RECURSIVA PARA INSERTAR UN APTAMER DE ALTA AFINIDAD EN EL ESPACIADOR MENOR DE CALMODULINA de *Trypanosoma cruzi.*

Resumen

Trypanosoma cruzi es el parásito protozoario causante de la enfermedad de Chagas, una enfermedad endémica en Panamá. En este parásito, la regulación de la expresión génica es principalmente un proceso postranscripcional. Se ha sugerido que las proteínas de unión al ARN tienen un papel clave en esta regulación génica. La calmodulina es una proteína sensora de calcio altamente conservada, codificada por tres genes separados por dos espaciadores intergénicos (espaciador mayor y menor). El estudio de las bases moleculares que llevaron a las interacciones ARN-proteínas en *T. cruzi* podría contribuir a comprender la biología del parásito para desarrollar estrategias novedosas para el control de enfermedades. Desarrollamos una metodología basada en una PCR recursiva para insertar un aptámero de alta afinidad de estreptavidina en la secuencia del espaciador menor, como base para el desarrollo de un sistema de captura de proteínas. La secuencia completa del locus de calmodulina se descargó de GenBank (AAHK01001263.1). La secuencia del aptámero reportadas en publicaciones anteriores (83 pb) se manipuló para el diseño del cebador. Las secuencias se editaron con el software bioinformático UGENE. La secuencia del espaciador menor de calmodulina (CMS) se fragmentó en dos regiones. Los cebadores se diseñaron para flanquear las dos regiones, cada uno con los oligonucleótidos internos directo / inverso unidos a secuencias de medio aptámero. La PCR recursiva final pudo amplificar el espaciador menor de calmodulina incorporando el aptámero. La secuencia fue confirmada por secuenciación de Sanger. La PCR recursiva representa una herramienta útil para insertar secuencias de alta afinidad como aptámeros para estudiar interacciones proteína-ARN específicas.

Palabras clave
Trypanosoma cuzi, Aptámero, PCR recursiva.

Introduction

Chagas disease is a neglected tropical disease caused by the protozoan parasite *T. cruzi*. It is estimated that 6-7 million people have been infected with *T. cruzi* and 70 million persons live at risk of infection in Latin America. Also, Chagas disease causes around 7500 deaths every

year (Stuart K. et al. 2008; Tzizik D. et al. 2018). In the post-genomic era, genomic and transcriptomic information could be used to discover new target proteins or pathogen-specific drugs (Crowther G. et al. 2010). This is the case of *T. cruzi* that has many published genomes from hybrid strains and particular linages and at least 186,461 nucleotide sequences available in the GenBank. The protein coding genes in this parasite are organized as a large array of consecutives genes throughout its genome that could be or not functionally related (El-Sayed et al. 2005). In this sort of array, the copies of multicopy genes are separated by intergenic splitters that might show differences in size and nucleotide composition (Liang X. et al 2003; El-Sayed et al. 2005). The transcription of these genes is carried out by RNA polymerase II which generates a large polycistronic transcript that resembles that produced by prokaryotes with the difference that the genes in the precursor RNA may not be functionally related (El-Sayed N. et al. 2005; De Gaudenzi J. et al. 2011). Unlike other organisms, no promoters for RNA polymerase II have been found in *T. cruzi* so the regulation of gene expression is thought to be entirely post-transcriptional (Clayton 2002; Sabalette K. et al. 2019).

The parasite circulates in two hosts, one invertebrate (triatomines) used as a vector and the other vertebrate (mammals) to complete its life cycle. (Bern C. et al. 2007; Chagas, C. 1909).
This alternation of hosts leads *T. cruzi* to undergo a process of cellular differentiation that produces specific stages that aid it to cope with and survive specific environmental variations (Onyekwelu K. 2019). To fulfill this phenotypic plasticity the parasite uses the post-transcriptional control to modulate the gene expression of more than 12,000 genes. In this context, morphological changes are followed by an adjustment in genetic expression which in the absence of RNA polymerase II promotors might be performed at specific post-transcriptional checkpoints (Das A. et al. 2017; Sabalette K. et al. 2019). Specific biological process as polyadenylation, translation, degradation of precursor RNA and mRNA degradation might act as specific transcriptional checkpoints (Clayton 2014).

Most of the studies on RNA-binding proteins associated to gene expression regulation have focused on the identification of proteins that bind to the untranslated regions (UTRs) of trypanosomatids genes (Cassola & Frash, 2009; Romaniuk et al. 2016; Ruiz E. et al 2018). Regulatory motifs found in *T. cruzi* UTRs interact with specifics RNA-binding proteins to decide the final destiny of mRNA and/or modulate the individual mRNA levels during *T. cruzi* life cycle (De Gaudenzi et al. 2003). As far as we know, no RNA-binding proteins that interact with specific motifs throw the polycistronic RNA precursor of tripanosomatids mRNA have been reported so far (D'Orso et al. 2003). The study of RNA-protein interaction at this level could shed light on the mechanism used by the parasite to control gene expression of protein coding genes in this transcriptional checkpoint.

Calmodulin is a highly conserved protein that act as a calcium sensor controlling in this way many essential intracellular processes in eukaryotic organism (Cheung 1980). It is not surprising, therefore, that a distortion of cellular calcium homeostasis due to a lack of this protein leads to cell necrosis and apoptosis (Zhivotovsky & Orrenius 2011). In *T. cruzi*, calmodulin is involved in important processes such as transport across the plasma membrane, cell growth and differentiation as well as in processes mediating intracellular signaling important for the parasite proliferation (Benaim et al. 1991; Souza et al. 2009). The genes coding for this protein, like other essential genes of this parasite (El-Sayed et al. 2005), are organized in a tandem array of gene copies separated by intergenic spacers showing different size and composition (Chung & Swindle 1990). The calmodulin locus of clone CL-Brener encompasses three gene copies separated by a major spacer of 1338bp and a minor one of 702bp. This fact makes calmodulin locus an excellent candidate to study specific interactions happening in the calmodulin spacers between gene copies located in the polycistronic pre-mRNA and *trans* elements like RNA-binding proteins in this specific transcriptional checkpoint. Herein, we developed a recursive PCR approach to insert in a specific point of the minor calmodulin spacer a high streptavidin affinity aptamer. This approach will permit us to capture specific RNA binding proteins that interact with motif located in the calmodulin

spacers. Approach like the one presented here could be used as a baseline to study how protein gene expression regulation is accomplished in specific gene locus of a particular polycistronic pre-RNA of any trypanosomatid.

MATERIALS AND METHODS

Cell culture and DNA extraction:
Epimastigote stage of *T. cruzi* clone 1551A5 were cultivated in LIT medium supplemented with 10% of fetal bovine serum. Only parasite cultures in the exponential phase were used to perform all experiments (Camargo E. 1964). Parasites cultures were centrifuged at 10,000g for 10 minutes and the pellet washed three times with PBS buffer using the same centrifugation speed. Afterward, the pellet was resuspended in 300µl of PBS and genomic DNA was isolated using the commercial kit Wizard Genomic DNA purification (Promega, USA) following manufacturer´s instruction. The DNA integrity was assessed by electrophorese in 0.8% agarose gels. DNA samples were quantified by fluorometric quantification using Qubit 4 fluorometer (Thermo Fisher Scientific – US).

Amplification of the minor spacer
To amplify the minor spacer of calmodulin locus we used the set of primers 5utrcal: 5'- GGAGATCTGCTCGTTGGACA-3 'and 3utrcal: 5'-GGTCAAATCAACTACGAGGA-3' which have been used earlier to obtain the spacers located in the calmodulin locus (Brandao & Fernández 2006). 50µl PCR reaction contain Platinum SuperFi taq polymerase (1U), dNTPs (200µM), $MgCl_2$ (3mM), 10µl of 5x high fidelity buffer, 150 ng of genomic DNA and nuclease free water. PCR conditions was set to: 96ºC for 3 minutes, and 35 cycles of 96ºC for 10 seconds, 55ºC for 30 seconds, 72ºC for 60 seconds and a final extension of 72ºC for 7 minutes. PCR products were visualized in a 1.5% agarose gel stained with GelRed (Biotium, USA) in 0.5X of TBE buffer. PCR amplicons were purified using the commercial kit wizard SV gel and PCR clean-up system (Promega, USA) following manufacturer´s instruction.

RECURSIVE PCR

Constructing the Hybrid Aptamer-calmodulin minor spacer

After selecting the site for the introduction of the aptamer sequence we split the spacer sequence into upstream and downstream section between the aptamer insertion sites. We performed two independent PCR reactions to amplified both sections and one final PCR to obtain the hybrid aptamer-calmodulin minor spacer by recombination. Primers used in this molecular approach are depicted on Table 1.

PCR conditions to obtain the downstream section

To amplify the downstream section, we used primers 3UTRcal and Aptamer1 at a final concentration of 0.3µM. The PCR reactions were performed in a final volume of 50µl containing dNTPS (0.2 µM), MgCl2 (1.5mM), Platinum SuperFi Taq polymerase (1U), and 5µL of 5x super Fi Buffer, 5µL of enhancer solution, 1µL of the purified minor spacer product and nuclease free water to complete 50µL. The downstream fragment was amplified after the following PCR conditions: an initial denaturation at 96°C for 5 minutes followed by 30 cycles of 96°C gradient for 30 seconds, 56 °C for 20 seconds and 72°C for 30 seconds and a final extension at 72°C for 7 minutes.

PCR conditions to obtain the upstream section

To obtain the upstream fragment of the calmodulin minor spacer we used primers 5UTRCal and Aptamer2 at a final concentration of 0.3µM. This PCR fragment was amplified in a final volume of 50µl containing the same reagents concentrations as the PCR mix used to amplify the downstream fragment of calmodulin spacer mentioned above. The PCR conditions to amplify this segment were an initial denaturation at 96°C for 5 minutes followed by 30 cycles of 96°C for 30seconds, 60°C for 20 seconds and 72°C for 30 seconds followed by a final extension at 72°C for 7 minutes.

Recombination of the upstream and downstream segment by PCR

After purifying, upstream, and downstream PCR products were used as molecular targets in a PCR reaction that aim to insert the S1 aptamer into the sequence of the minor spacers. To do so, we use the primers

3UTRCal and 5UTRCal and the same PCR reagents concentration used to amplify the calmodulin minor spacer described above. The PCR conditions consisted of an initial denaturation at 96℃ for 5 minutes, 10 cycles of 96℃ for 15 seconds, 55℃ for 60 seconds and 72℃ for 30 seconds followed by 35 cycles of 96℃ 10 per second, 56℃ for 30 seconds, 72℃ for 30 seconds and a final extension of 72℃ for 5 minutes. Figure 4 shows the amplified products of the upstream and downstream fragment as well as the amplification product obtained by recombination.

Cloning of Amplified Products:
To clone amplified products, we used the commercial kit pGEM®-T Easy Vector kit (Promega, USA) following the manufacturer´s instructions. Around 1 to 3µl of amplifications products were used in the ligation reactions that were carried out at 4℃ overnight. Two microlites of cloning reaction were used to transform competent cells DH5alpha. The plasmid was added to the competent cells by heat shock at 42°C. The white/blue screening to obtain plasmid with inserts was carried out in LB plates with ampicillin (100µg/ml) and X-gal (40µg/ml). At least twenty white colonies were selected and culture in LB medium to recover plasmids using Wizard® Plus SV Minipreps DNA Purification System kit (Promega, USA) according to the manufacturer's recommendations.

Sanger Sequencing
Plasmids obtained from each clone were sequenced using the BigDye Terminator v3.1 kit (Applied Biosystems) following the methodology described by the manufacturer. To carried out the sequencing reaction, we used the primer set SP6: 5'- TATTTAGGTGACACTATAG-3´and T7 5`-TAATACGACTCACTATAGAGGG-3` and 50ng of the plasmid. Capillary electrophoresis of all sequenced products was performed and analyzed by the genetic analyzer 3500XL (ThermoFisher Scientific, USA). The electropherograms editions and the identification of inserts was performed using the software UGENE (Okonechnikov et al. 2012).

RESULTS AND DISCUSSION

We designed a molecular approach to insert the Streptavidin high aptamer (S1) into the middle of the minor spacer of calmodulin locus. *T. cruzi* (clone CLBrener) Calmodulin gene (including major and minor spacer) were obtained from GenBank (accession number XM_802998.1, XM_802996.1 and XM_802995.1). We were able to identify all elements of calmodulin locus and design all primers set necessaries to add the S1 aptamer into the minor spacer by using the bioinformatic package UGENE version 1.32.0. Also, we simulated the secondary structure of the minor spacer using the software Mfold version 2.3 (Zucker, 2003) before and after the insertion of the S1 aptamer in several points of the minor spacers until finding the site of insertion where no disruption of the secondary structure of the minor spacer takes place (Figure 1 and 2). After this primary evaluation, we designed a PCR approach based upon a recursive PCR, method described for the first time by Walker et al. 2008 while constructing a synthetic gene using this molecular technique.

Regarding protein isolation, Streptavidin-binding aptamers offers several advantages over other methodologies. For example, it offers a simple one-step purification methodology without needing recombinant protein production, matrixes readily available, and the option of labeling *in vitro* transcribed RNA or cell-expressed RNA (Leppek & Stoecklin, 2014).

Indeed, recursive PCR is becoming one of the techniques of choice to recombine other sequences into selected amplicons. Also, using this approach in strategies aiming to capture specific proteins by methodologies like “pull down” would help to uncover specific molecular targets that could be used in the control of this neglected disease.

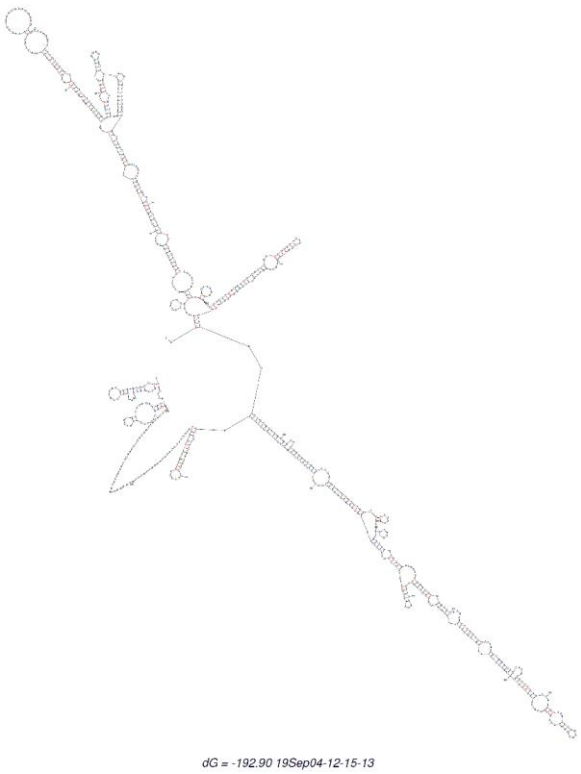

Figure 1. Putative Secondary structure of the minor spacer of the calmodulin locus estimated by the software Mfold.

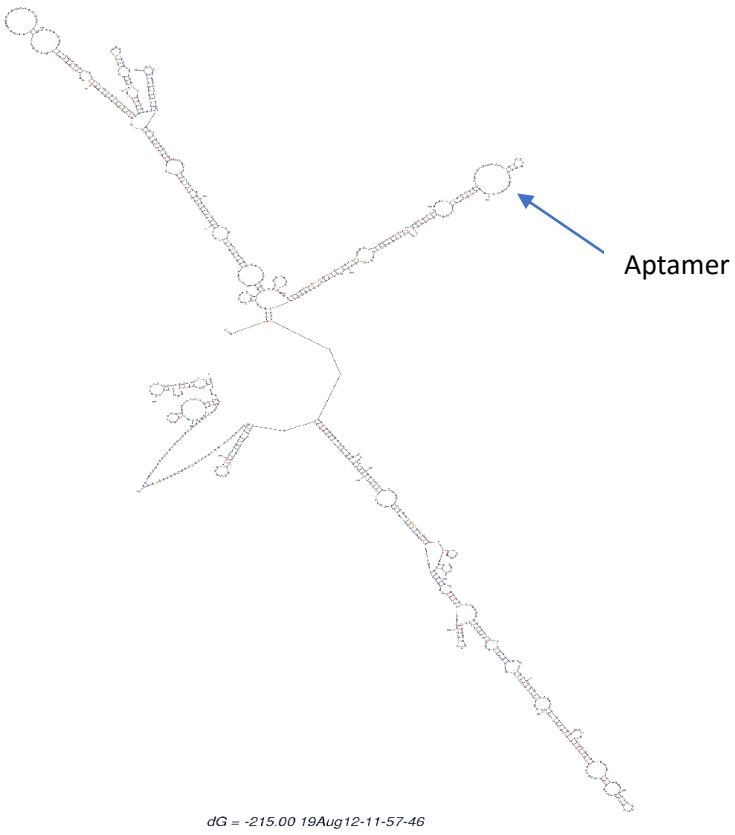

Figure 2. Insertion position of the Aptamer at base 312 of the calmodulin spacer estimated by the software Mfold.

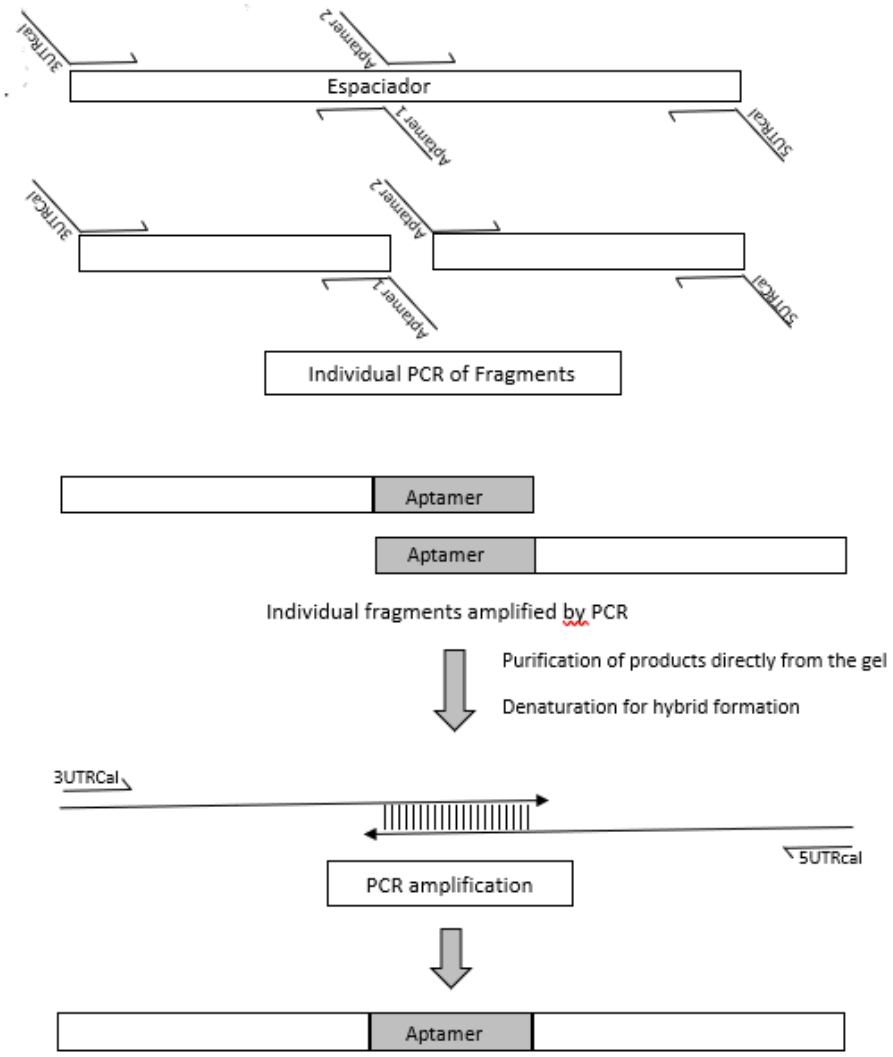

Figure 3. Approach used to insert Aptamer S1 at the bases 312 and 492 of the calmodulin minor spacer by recursive PCR.

Table 1. Primers used in this study.

Name	Sequence	Position in the spacer or ORF
5UTRcal	GGAGATCTGCTCGTTGGACA	ORF
Aptamer1	**CCGGCCCGCGACTATCTTACGCACTTGCATGA TTCTGGTCGG**TGGGCGCAGCTCAGCTGT	295-312
Aptamer2	**CCGACCAGAATCATGCAAGTGCGTAAGATAG TCGCGGGCCG**GTGGAGCAGAGAAGGAGGTTG	355-354
T73UTRcal	**TAATACGACTCACTATAGGG** GGCCAAATCAACTACGAGGA	ORF

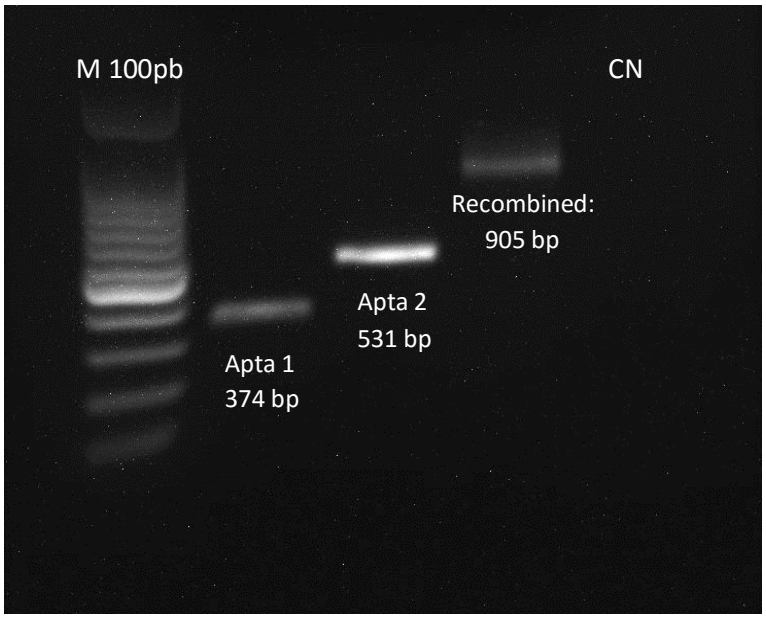

Figure 4. PCR products obtained by the recursive PCR. Apta1: downstream amplicon; Apta2: upstream amplicon; Recombined: amplification product obtained by recombination.

CONCLUSION

Recursive PCR allowed the incorporation of a high affinity streptavidin aptamer into the minor spacer of the calmodulin locus of *T. cruzi*. Recursive PCR represents a useful tool for the generation and recombination of fragments of interest that allow the study of protein-RNA interaction between sequence motifs and target proteins involved in important biological functions essential for the *T. cruzi* survival.

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