SHORT COMMUNICATION

Charaterization of *Leishmania major* Friedlin Telomeric Terminus

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Here we have characterized Leishmania major *(Friedlin) telomeric terminus (the very end) using recombinants obtained by a vector-adaptor cloning protocol. As in* L. donovani, *the last nine nucleotides of* L. major *terminus are 5'-GGTTAGGGT-OH 3', differing from* Trypanosoma cruzi *and* T. brucei *terminus 5'GGGTTAGGG-OH 3', thus indicating that these sequences are genus specific. We have also made a comparative analysis between* L. major *and* L. donovani *telomere-associated sequences, and described a novel non-repeated telomeric associated sequence common to* L. major *low molecular weight chromosomal bands.*

Key words: *Leishmania major* - telomeric terminus - cloning

In most eukaryotes telomere structure is conserved; a typical telomeric DNA sequence consists of tandemly arrayed G-rich repeats running 5' to 3' towards the end of the chromosome, ending in a single strand chain (overhang) of variable length. Phylogenetically unrelated organisms such as vertebrates and Kinetoplastida share identical telomeric repetitions $(TTAGGG)_n$, suggesting common basic mechanisms for generation and maintenance of telomeres.

The single strand nature of the overhang was first demonstrated in *Tetrahymena* and *Oxytrichia*, and later confirmed for most eukaryotes (Klobutcher et al. 1981). The size of the overhang is species specific, varying from 16 nucleotides in *Oxytricha* to 50-100 nucleotides in mouse and human (Klobutcher et al. 1981, Wright et al. 1997). The importance of the overhang is suggested by the fact that mutants disrupting the nature of the G-rich strand eliminate telomere function (Van Steensel et al. 1998).

The overhang is a dynamic structure, interacting with other overhangs, specialized proteins, and the nuclear membrane (Henderson 1995). In mammalian cells (Griffith et al. 1999), and recently in *Trypanosoma brucei* (Muñoz-Jordán et al. 2001), it has been demonstrated that telomeres have terminal loops, or T-loops, where the single strand region is tucked back inside the double-stranded portion of the telomere, stabilized by specialized proteins, thus protecting the chromosome terminus.

The subtelomeric region consists, of unique sequences, or repetitive sequences with variable lengths and degrees of repetitiveness which are often species-specific (Henderson 1995). Some of these sequences are restricted to the telomeres and participate in telomeric function. Chromosomal rearrangements at subtelomeric and telomeric regions have been implicated in various phenomena ranging from chromosome length polymorphisms in *Leishmania* sp., to the generation of antigenic variants in *T. brucei* and *Plasmodium falciparum* (Corcoran et al. 1988, Ravel et al. 1995, Rudenko et al. 1996). Recently, Sunkin et al. (2000) have shown that size differences between *L. major* chromosome 1 homologues is largely restricted to variation of both the number and content of sub-telomeric repetitive elements, suggesting intra-chromosomal rearrangement events.

In previous works we demonstrated (Chiurillo et al. 1999, 2000) that telomeres can be cloned by complementing the last nine nucleotides of the overhang with an adaptor sequence. With this procedure there is no need to alter the overhang, and the phase of the sequence of hexameric repeats can be deducted. Using this protocol, we have here cloned and characterized telomeric sequences of *L. major* Friedlin (MHOM/IL/81/Friedlin), the selected organism of the *Leishmania* Genome Sequencing Project (Bastien et al. 1998).

To select the restriction enzyme for cloning, *L. major* genomic DNA was digested with endonucleases *Pst* I, *Rsa* I and *Sau* 3AI, and the corresponding Southern blots were hybridized with radiolabeled telomeric oligonucleotide $(CCCTAA)_3$ (not shown). From these experiments *Pst* I was chosen because the size of the fragments produced (2-12 Kbp) was convenient for cloning in pBluescript. In addition, the presence of a unique *Pst* I cutting site within this plasmid allowed us to ligate the vector-adaptor to *Leishmania* high molecular weight DNA prior to restriction digestion. Thus by reducing the number of unspecific fragment ends, we expected to increase the probability of successful hits between the adaptor and the telomeric overhang. The rest of the cloning protocol was done as described by Chiurillo et al. (1999).

Three hundred and ninety white colonies were screened with a radioactive telomeric probe, out of which, 23 were positive. Insert size of a randomly selected group of recombinants ranged from 0.85 to 4.5 Kbp.

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Nine of these recombinants (LmFtel) were partially sequenced with T3 and T7 primers using an automated ABI 377 instrument (Cesaan IVIC). Computer analysis was done with a DNAMAN 3.2 software (Lynnon BioSoft).

Nucleotide sequences reported in this paper are available in the GenBank™ database under accession numbers: AY014832-35.

Sequence readings from T3 primer side (Fig. 1) showed that the last nine nucleotides of all recombinants complemented the adaptor with the sequence 5'-ACCCTAACC-OH 3', followed by variable numbers of 5'-ACCCTA-3' repeats.

Three recombinants presented hexameric repeats followed by two sequence blocks of 102 bp each. These blocks, or LCTAS (*Leishmania* Conserved Telomere-Associated Sequence), were first reported in *L. major* by Fu and Barker (1998). Fig. 2A shows a sequence comparison of *L. major* and *L. donovani* LCTAS; the former had 4 to 5 copies of imperfect octamers and a 62-bp sequence (Chiurillo et al. 2000), whereas *L. donovani* shows variable numbers (1-11) of a well defined octamer repeat (5'-TGGTCATG-3'), and a 62 bp sequence (Fig. 2A) (Chiurillo et al. 2000). Sequence specificity and high copy number of *L. donovani* LCTAS have been exploited in the design of a PCR assay that detects *L. donovani* and *L. infantum* with high sensitivity (Chiurillo et al. 2001).

Fig. 1: summary of the organization features of *Leishmania* telomere terminus. Uppercase letters represent telomeric hexamer repetitions (in brackets the number of repeats found in this work) and the first nine nucleotides of the overhang (underlined). Dotted line indicates that the overhang could be longer than nine nucleotides. LCTAS: *Leishmania* Conserved Telomere-Associated sequence; NRTAS: non-repeated telomeric-associated sequences

Eight recombinants (Table) exhibited a 781 bp non-repeated telomeric associated sequence (NRTAS781) not previously reported in GenBank (AY014835). This sequence contained a *Pst* I site that, combined with pBluescript insert capacity, may explain its preferred cloning. In this group of recombinants two had two LCTAS copies.

The presence of LCTAS in some recombinants confirms prior observations that *L. donovani* and *L. major* have two types of telomeres, those with hexameric repeats followed directly by NRTAS, and a second type with LCTAS blocks inserted between the terminal hexamers and the NRTAS (Fu & Barker 1998, Chiurillo et al. 2000).

When *L. major* chromoblots were probed with LmFtel 2A11-NRTAS (devoid of hexamers or LCTAS) most of the bands recognized (except for the compression and the

TABLE

Leishmania major Friedlin telomeric clones

Recombinant LmFtel	Size (Kbp)	Hexamers [a]	LCTAS	NRTAS
1E8	4.50	19	2	1E8
2A11	0.85	12	-	781
3A11	1.18	7	2	781
3C4	1.21	8	2	781
3G3	0.85	12	-	781
4A3	0.90	10	-	781
4A7	0.91	17	-	781
4B6	0.91	17	-	781
4D10	0.85	12	-	781

LCTAS: *Leishmania* Conserved Telomeric-Associated sequence; NRTAS: non-repeated telomeric-associated sequence; *a*: number of hexameric repeats preceding the first LACTS or NRTAS

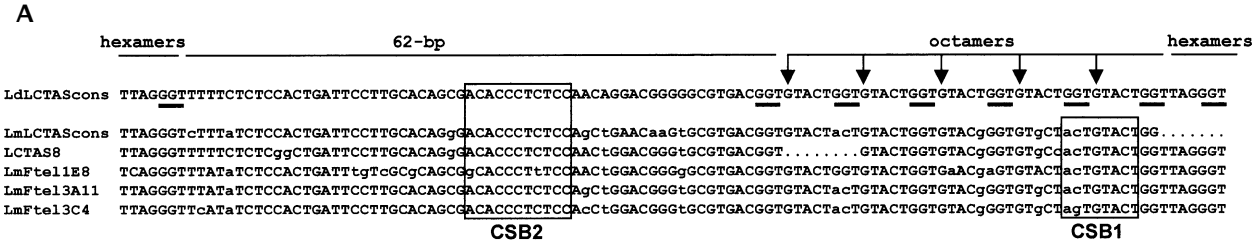

Fig. 2: sequence alignments of *Leishmania* Conserved Telomere-Associated (LCTAS). A: arrows mark octamer repeats. Nucleotide differences between *L. donovani* and *L. major* octamer and 62-bp sequences are represented in lowercase letters. Conserved sequence boxes (CSB) are included in squares. LdLCTAScons is a consensus sequence derived from *L. donovani* (Chiurillo et al. 2000), LmLCTAScons and LCTAS8 are *L. major* consensi sequences reported by Fu and Barker (1998). Triplets GGT in LCTAS and between LCTAS and non-repeated telomeric-associated sequences (NRTAS) are underlined; B: triplets in the interphases NRTAS-LCTAS of two *L. major* telomeric recombinants (underlined).

well) were in the low molecular weight range, including the two bands corresponding to chromosome 1 homologues (Fig. 3, arrows). NRTAS781 was not reported in the complete sequence of chromosome 1(AE001274) (Myler et al. 1999), and we suspect that it was missed during sequence reconstruction of one of the telomeres. As these authors stated, the sequence of one chromosomal end was derived from a chimerical cosmid containing parts of two different chromosomes (other than 1) (Myler et al. 1999, Sunkin et al. 2000).

Clone LmFtel 1E8 (the ninth recombinant) had two LCTAS blocks followed by a NRTAS (NRTAS1E8) that has been previously found in *L. major* (AF031202) and *L. donovani* (AF095768). The clone had a 4.5 Kbp insert with 320 bp made of hexamers and LACTS. DNA readings from the T7 side revealed a non-coding sequence with no homology in GenBank database (AYO14832).

As found in *L. donovani* telomeres (Chiurillo et al. 2000), the first three nucleotides of the overhang ACC/GGT are present in the transitions between hexamers and LCTAS blocks (prior or after), and hexamers and NRTAS (Figs 1, 2A, B). These similarities suggest a common origin of these sequences. An equal situation is found in *T. cruzi*, *T. brucei* and *Giardia*. In *Trypanosoma* the first nucleotides of the overhang (5'-GGGTTAGGG-3') are GGG (Beck 1997, Chiurillo et al. 1999), which is the same trinucleotide connecting the last hexamer with the unique subtelomeric sequence. In *Giardia* (Adam 1992) all rDNA/telomere junctions have the sequence CCCCGG, which contains the first three nucleotides of *Giardia* telomeric repeat (CCCTA). It has been suggested that this junction could be the healing site of chromosomal breakage (Adam 1992). Although no chromosome rupture phenomena has been documented in Kinetoplastida, it is likely that if such an event takes place, the trinucleotides described could provide templates for telomerase elongation or any other chromosome healing mechanism.

In ciliates, primary sequence and biochemical experiments indicate that *Oxytricha* and *Stylonychia* telomerase RNA template 5'AAAACCCC-3' is permutated in *Euplotes aediculatus* to 5'-CAAAACCC-3' (Lingner et al. 1994). Although Kinetoplastida telomerase activity has been demonstrated (Cano et al. 1999), no telomerase gene has been cloned and little is known about its regulation. However, from the evidences of this work we would like to suggest that *Leishmania* telomerase adds 5'-TAGGGT-3' blocks to the growing telomeres, and from results of other works, *Trypanosoma* (Beck 1997, Chiurillo et al. 1999) adds 5'-TTAGGG-3' blocks, the same but permutated sequence. Thus a divergent evolution in end-processing mechanisms may have occurred between the two genera. Possibly the precursor of the Trypanosomatidae family suffered a shift change in the RNA template region that caused this divergence.

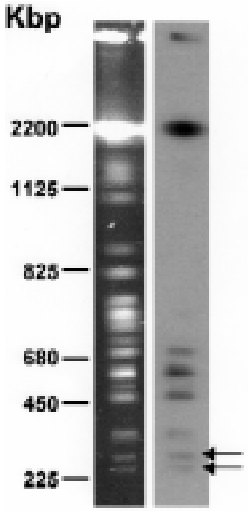

Fig. 3: *Leishmania major* chromoblot hybridized with a NRTAS781-derived probe. A restriction fragment (550 bp) isolated from recombinant LmFtel 2A11 was radioactively labeled with dCTP α^{32}P by random primer (Megaprime DNA labeling system/Amersham), and used as probe against a chromosomal blot of *L. major* CHEF separated chromosomal bands. Left, ethidium bromide stained CHEF gel. Right, autoradiograph after hybridization with the probe. MW maker size (right) was based of Yeast chromosomes used as standard. Arrows to the right point to chromosome 1 homologues bands. The nylon filter was hybridized at 65ºC in 0.5 M Na-phosphate pH 7.2, 7% SDS, 1 mM EDTA, 0.1 mg/ml *Escherichia coli* tRNA for 18 h. After hybridization, the filter was washed twice at room temperature with 40 mM Na-phosphate, 0.1% SDS for 15 min, and twice with the same buffer at 65ºC for 15 min.

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