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A New Class of *Leishmania tropica* Minicircle Mitochondrial DNA

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Abstract—A new class of mtDNA minicircle molecules with a circular length of 430 bp, i.e., approximately half of the ordinary mtDNA, was detected by electron microscopy in *Leishmania tropica*. Some characteristic structural elements of mtDNA, i.e., conservative and variable region including the presumable guiding RNA gene, were detected in the nucleotide sequence of minicircle molecules, as well as CCAAT and TAGTTTGA sequences which may function as regulatory elements in RNA editing. Minicircles may serve as operative copies of standard molecules in RNA editing.

Key words: kinetoplast DNA, protozoans, RNA editing, mitochondrial genome

INTRODUCTION

RNA editing in trypanosomal mitochondria involves addition or excision of uridine residues and produces mature mRNA from unedited precursors [1]. This process is supposed to be one of the mechanisms controlling parasite–host interactions, molecules of guiding RNA (gRNA) playing a key role in it. Editing is presumably initiated by the formation of hybrid molecules between 5'-terminal sequences of gRNA and mRNA sequences located at the 3' end of the edited region [2]. Many details of this process still remain obscure and need experimental verification. By now, several gRNA encoded in maxicircle molecules have been investigated [3]. It should be taken into account that kinetoplast DNA (kpDNA), alongside with 20–50 maxicircles, contains $5 \cdot 10^3$–$5 \cdot 10^4$ minicircles per cell [4]. The genetic function of minicircles remained vague until the *Leishmania tarentolae* minicircles were studied [5]. It appeared that they code for gRNA participating in editing of maxicircle cryptogenes. Moreover, it was found that the bulk of gRNA is encoded in the minicircle component of kpDNA. Conservative and variable regions were detected in minicircles [6]. In *Trypanosoma* and *Leishmania* species the conservative region comprises approximately 10% of the molecule [7]. The number of gRNA genes therein is variable. For example, in *T. brucei* minicircles carry three transcribed gRNA genes located in the variable region between 18-mer inverted repeats [8], while all the classes of *Leishmania* minicircle kpDNA studied to date have but one gRNA gene. The number of classes of minicircle molecules with a specific primary structure differ in the species studied. For example, 10–20 classes were found in *L. tarentolae*, 10 in *Phytomonas davidii*, while more than 300 classes of minicircles were identified in *T. brucei* [4, 9, 10]. Until recently, all the classes of minicircles of a species of *Leishmania* genus seemed to be homogeneous in size but varying in primary structure [6].

EXPERIMENTAL

Strains K-27, TAT-3, MRCB-JBF, NLB-297, and ROSSI-II were studied; they are briefly characterized in the table. The yield of total DNA was 1–3 μg per 10^8 cells. Use was made of restriction endonucleases from Boehringer Mannheim, phage T4 DNA ligase from Promega, DNA fragments isolation kit USBio-Clean from Amersham, recombinant DNA purification kits from Promega (Wizard Minipreps DNA purification system) and Qiagen, and radioactive dNTPs from Amersham-USB.

For molecular cloning, pBlueScriptKS+ and *E. coli* strain XL1Blue were used. PCR products were cloned using *Hin*dIII and *Bam*HI sites inserted in the primer terminal sequence.

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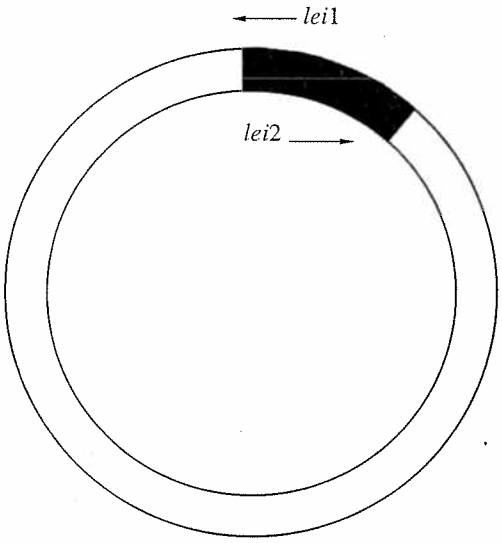
Strains of *L. tropica* studied

Strain	Parasitological code
K-27	MHOM/SU/74/SAF-K-27
TAT-3	MHOM/TU/85/TAT-3
NLB-297	MHOM/KE/84/NLB-299
MRCB-JBF	MRAT/IQ/72/MRCB-JBF
ROSSI-II	MHOM/NA/76/ROSSI-II

DNA sequences were determined by the Sanger method using double-stranded templates and Amersham-USB and Life-Technologies kits for direct sequencing of PCR products.

The PCR mixture was kept at 94°C for 5 min and then subjected to 30 amplification cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min) with final incubation at 72°C for 10 min, in Perkin-Elmer or Cyclotemp 5 units; the primers were *lei*1 (5'-TTAAGCTTGGTGTAAAATAGG-3') and *lei*2 (5'-TTGGATCCAACCCCTAGTT-3').



lei1 TTAAGCTTGGTGTAAAATAGG →
GGCCTCCCCGTGCACAACTAGGGGTTGGTGTAAAATAGGG
CCGGAGGGGCACGTGTTGATCCCCAACCACATTTTATCCC
←TTGATCCCCAACC*TAGGTT* *lei2*

Fig. 1. Structure of *L. tropica* minicircle, with the *lei*1 and *lei*2 primer annealing sites inside the conservative region (filled part), arrows show the direction of amplification. Bottom, fragment of the conservative region with the primer annealing sites.

Electron microscopy was done according to Davis *et al.* [11].

DNA sequences were analyzed using DNASIS (Hitachi Software Engineering Co. Ltd), GeneBee, PCGENE-6.5 (IntelliGenetics Inc. and Genofit SA, 1991), DNASTAR for Windows (ver. 3.03, 3.04, 1993–1995), and NCBI-BLAST program packages.

RESULTS AND DISCUSSION

When *Leishmania* minicircle kpDNA polymorphism was analyzed by PCR, it was found that primers annealing at the universal dodecamer (GGGGTTGGTGTA) of the conservative region (Fig. 1) yield three products on total DNA (Fig. 2). The length of the largest product determined by restriction endonuclease analysis, electron microscopy, and direct sequencing appeared to be 900–920 bp; it corresponded to the full-length *L. tropica* minicircle. The second product, approximately 450 bp long, was present in minor quantities.

To check the possibility that the second and the third product result from nonspecific interaction of the primer with minicircles, the annealing temperature during PCR was varied from 50 to 61°C, but the patterns obtained after electrophoresis did not change (not shown). Additional amplification of individual products extracted from gels demonstrated that the smaller products are not subproducts of the larger ones (Fig. 3), thus excluding the possibility that additional sites capable of interacting with primers are present in full-length molecules. Such sites also could not be found in the full-length minicircle kpDNA.

The 430-bp product was sequenced (Fig. 4). This figure also shows a fragment of *L. tropica* full-length minicircle and of the PCR product. Comparison of the sequences reveals that, first, a region identical to the standard conservative sequence is present in the analyzed fragment. Second, the molecule contains a fragment completely identical to the sequence of a gRNA gene of *L. tarentolae* and *T. brucei*, *NDIII*.

The very high homology of *Leishmania* gRNA genes, which could be traced even over Trypanosomatidae, provokes interest. It may be explained by high evolutionary conservatism and the prominent functional role of these genes.

The CCAAT and TAGTTTGA sequences found in the analyzed molecules may play a regulatory role in RNA editing. As total DNA was used in PCR experiments, the nature of corresponding templates and their localization in the cell remained obscure. In an attempt to understand the nature of templates, *L. tarentolae* cells were disintegrated, nuclei were harvested and used to isolate DNA. DNA samples were then sub-

Fig. 2. PCR products synthesized on total *L. tropica* DNA. Electrophoresis in 1% agarose gel, TAE buffer.

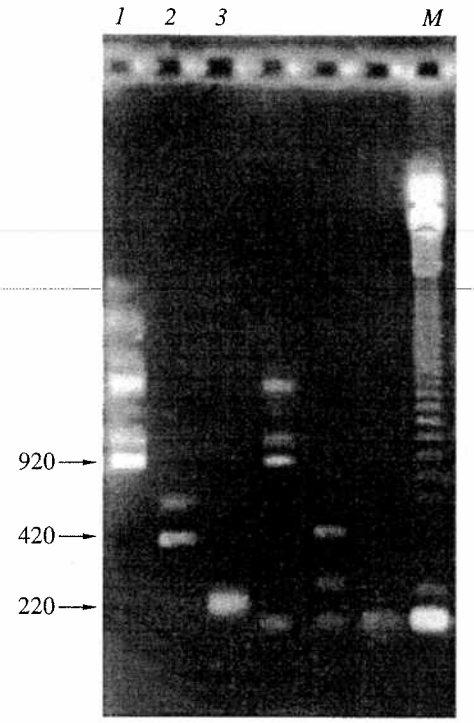


Fig. 3. Electrophoretic separation of products of repeated amplification (see text) of specific zones: (*1*) full-length minicircle, (2) 430 bp, and (*3*) 200 bp; (*M*) 123-bp size marker (Life Technologies); 1% agarose gel, TAE buffer.

jected to electrophoresis in agarose gels, parts of the gels with DNA fragments 400–600 bp long were eluted and PCR-amplified. Identical samples were studied by electron microscopy. Mobility of the PCR product formed in these conditions appeared to be indistinguishable from that of the product obtained with total cell DNA as template. Electron microscopy confirmed that the extracted DNA contains circular molecules 420–500 bp long. As expected, these molecules constituted the minor component.

It should be noted that the analyzed product is not formed in PCR if purified kpDNA associate is used as template. One may conclude that these molecules are not the associate component, but exist independently.

Summing up, one may conclude that a minor class of minicircle DNA including the complete conservative region and one gene of gRNA, and constituting not more than 3–5% of total mtDNA is present in *L. tropica* mitochondria. The size of these molecules is approximately one half of that of standard minicircles. The properties of DNA of this fraction allow a prediction that it may govern active transcription of gRNA genes. If so, the role of standard, "large" minicircles is to preserve gRNA genes, to replicate them and to transmit them from generation to generation, whereas the activity of gRNA is based on its fast transcription from "small" minicircles carrying a single gRNA gene. The small size and the absence of auxiliary nonfunctional sequences may facilitate and enhance transcription of gRNA genes. If this hypothesis is correct, several subclasses of minicircles may exist carrying different gRNA genes. The presumable functional importance of 430-bp minicircles is supported by the fact that the content of this component depends on the functional state of a protozoan culture.

Interestingly, similar PCR products are formed when closely related *L. infantum* kpDNA is used as

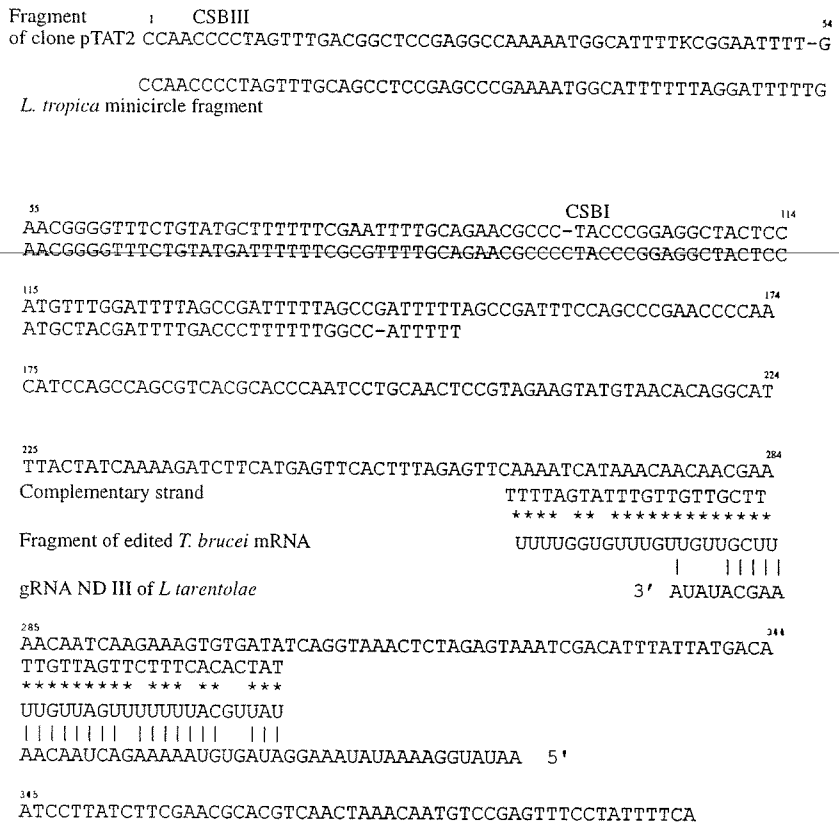


Fig. 4. Localization of gRNA gene in the 430-bp minicircle. For comparison, nucleotide sequences of a fragment of *L. tropica* full-length minicircle, of an edited *T. brucei NDIII* mRNA, and of *L. tarentolae NDIII* gRNA are given.

template. However, with *L. major* and *L. gymnodactyli*, PCR yields only one product with the molecular mass of the complete minicircle.

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