Leishmania tarentolae minicircles of different sequence classes encode single guide RNAs located in the variable region approximately 150 bp from the conserved region

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ABSTRACT

The complete sequences of three kinetoplast DNA minicircles (B4, D3 and D12) from *Leishmania tarentolae* are reported. All *L. tarentolae* minicircles encode single gRNAs localized within the variable region approximately 150 bp from the conserved region. The 5' termini and tentative 3' termini of the new gRNAs were determined and the gene sequences and flanking sequences of all minicircle gRNA genes compared for conserved motifs of possible transcriptional regulatory significance. All minicircle gRNAs possess 3' oligo-[U] tails of variable length similar to maxicircle gRNAs. A role for the D3 minicircle gRNA in the editing of the 5' pan-edited MURF4 mRNA was suggested by sequence analysis, and a role for the D12 minicircle gRNA in the editing of the COIII mRNA and another minicircle gRNA (Lt154) in the editing of the pan-edited G6 mRNA have been previously reported. The cryptogene mRNAs edited by the B4 and Lt19 minicircle gRNAs are yet undetermined.

INTRODUCTION

Guide RNA (gRNAs) are small RNA molecules which have been implicated to play a crucial role in the process of RNA editing in trypanosome mitochondria (1–7). The 5' ends of the gRNAs form hybrids with mRNA sequences immediately 3' of the pre-edited regions (PER), thereby initiating the editing process. After completion of editing, the gRNA and the mature edited mRNA form perfect hybrids over the entire length of the gRNA. Evidence for the involvement of gRNAs in the RNA editing process has been largely indirect, mainly through the sequence complementarities of gRNAs and mature edited sequences and analysis of presumed editing intermediates (8–10). The discovery in *L. tarentolae* of chimeric gRNA/mRNA molecules with the gRNAs attached to mRNAs at editing sites by the oligo-[U] tail (6) is consistent with a transesterification model for RNA editing,

in which the gRNA provides an internal guide sequence and functions as a source of the added uridines (6,11).

Seven conserved maxicircle-encoded gRNAs have been characterized from *L. tarentolae* (1) and *Crithidia fasciculata* (7) and three from *Trypanosoma brucei* (7). In addition to the 20–50 maxicircle molecules, the kinetoplast DNA (kDNA) network contains approximately 10^4 catenated minicircle molecules (12). The genetic function of the minicircles was unknown until the discovery in *L. tarentolae* that these molecules also encode gRNAs involved in editing of the transcripts of maxicircle cryptogenes (2). Minicircles are organized into conserved and variable regions (12,13). In *L. tarentolae* and *T. brucei* each molecule has a single conserved region representing approximately 10% of the molecule. The number of minicircle sequence classes that exist in a single network is species-dependent. In *L. tarentolae*, there are approximately 10–20 minicircle sequence classes, whereas, in *T. brucei*, there are over 300 sequence classes (12,13). Three complete *L. tarentolae* minicircle sequences have been reported (14). An additional five minicircle sequence classes from *L. tarentolae* were determined by partial sequence analysis of minicircles which were cleaved at specific sites with mung bean nuclease in 50% formamide (15). In this paper we present the complete sequences of three of these minicircle classes.

In *T. brucei*, each minicircle contains three gRNA transcriptional units precisely situated between 18mer inverted repeats (16) in the variable region (4). We show that, in *L. tarentolae*, each minicircle contains a single gRNA gene situated approximately 150 bp from the conserved region.

METHODS

Cell culture and purification of kDNA and kRNA

L. tarentolae cells (UC strain) were grown as described previously (17). Cells were harvested at mid log phase and used for mitochondrial isolation for the preparation of kinetoplast RNA

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as described (18), except that RNA was isolated from the enriched kinetoplast fraction without centrifugation through Renografin. Kinetoplast DNA was isolated from stationary phase cells as described (19).

Oligonucleotide probes and primers

Oligonucleotides were synthesized and purified as described (1). Oligonucleotide probes were 5' end-labeled with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ - 32 P]ATP (ICN) as described (1). The oligonucleotides used for PCR amplification and as probes are listed below. As a control for specificity, the oligonucleotide probes were hybridized to blots of digested kDNA and also used as primers for direct sequencing of minicircles from uncloned network DNA. In all cases the probes were specific for minicircle sequences (data not shown).

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kRNA was electrophoresed through 1.5% formaldehyde-agarose gels or 20% acrylamide gels and blotted onto Nytran nylon membranes. Hybridization and wash conditions were as described (2).

PCR amplification, cloning and sequencing of minicircles

L. tarentolae minicircles B4 (oligonucleotides S-276, S-277), D3 (S-278, S-279) and D12 (S-259, S-262) were amplified using primers based on known sequence (2,15). Amplified products were digested with EcoRI and BamHI and asymmetrically ligated into the pGEM-7Zf cloning vector as previously described (9). Several minipreps were prepared for each clone, and sequencing was performed as described (9) using the Sequenase Sequencing Kit (U.S. Biochemical).

PCR amplification and cloning of chimeric molecules

All chimeric amplifications were performed as described (6). PCR products were cloned directly or after size selection in acrylamide with the TA Cloning Kit (Invitrogen Corp.). Colonies

were screened by hybrid selection with the corresponding internal gRNA primers or sequenced at random.

Oligonucleotides used (non-encoded sequences added for restriction sites are in boldface):

S-255 (D12 gRNA probe, nt 322–341): GAATAGTGTTCATCTCTC
S-260 (D12 5' PCR, nt 326–345): ATATGGATCCGATGAAAACACTA-TTCGTAG

S-262 (D12 3' PCR, nt 327–308): GCGCGAATTCTCTGTCTTTACCTGCT
S-276 (B4 5' PCR, nt 408–424): GCGGAATTCTATAATCATTATCGTCC
S-277 (B4 3' PCR, nt 407–391): TATGGATCCAGGATAGCATAGACGGC
S-278 (D3 5' PCR, nt 167–150): GCGGAATTCAAGAAAATTCCTAAAA
S-279 (D3 3' PCR, nt 183–167): TATGGATCCACACATCTGGTGCACGT
S-303 (L19 gRNA, nt 561–580 relative to CSB-3)(20): TATTTTTCACCTC-AACCACA

S-310 (D12, nt 500–481): TCTATATCCATATATATTGC
S-311 (D12, nt 671–652): CAGCCCTACCTAGGCATATA
S-312 (D3 gRNA, nt 342–323): ACTCTACAATGTTCCCTATA
S-313 (B4 gRNA, nt 323–304): AATGTTTGTATATTCTCT
S-366 (L126, nt 653–672): GCGGAATTCTAAGCCAGATGCATTTTCAG
S-367 (L126, nt 652–633): TATGGATCCTAGACCAGTGCTCAGTAGCG

RESULTS

Three new kDNA minicircle sequence classes

Three specific minicircles were PCR amplified from total kDNA using oligonucleotides based on partial minicircle sequences from the published mung bean nuclease-cleaved minicircle fragments D3, D12 and B4 (15). The full length minicircle products were cloned and sequenced. The complete sequences are shown in Fig. 1A, 1B, and 1C. The nucleotides are numbered ending with the universal conserved CSB-3 12mer sequence block (14,20), which represents an origin of replication for one strand (21).

Relationship of the D3 minicircle sequence to the L126 minicircle sequence

The D3 sequence in Fig. 1 is closely related in sequence to the L126 minicircle sequence reported previously (14), but there are multiple gaps in both sequences and one large insertion in the

Figure 1. Complete sequences of three minicircles from *L. tarentolae* kDNA. The transcribed gRNAs are double-underlined and the 5' ends labeled. The tentative 3' ends are indicated by the underlining. The conserved regions (intra-species) are indicated by shadowing and the CSB-3 12mer boxed and labeled. The T's complementary to the phased runs of A's giving rise to the bend are underscored with wavy lines. (A). Sequence of the B4 minicircle. The partial B4 sequence previously reported (15) covers nt 348–746, but shows several differences with the present sequence, especially between B4 nt 616–800. (B). Sequence of the D3 minicircle. The partial D3 sequence previously reported (15) covers nt 56–346. The arrows indicate the two observed 3' ends, as determined from chimeric molecules. The A's are indicated since it is impossible to tell if the terminal T (= U) is encoded or part of the oligo-[U] tail. (C). Sequence of the D12 minicircle, with additional oligonucleotide probes boxed. The partial D12 sequence previously reported (15) covers nt 256–465.

D3 sequence. An attempt to PCR amplify the Lt26 minicircle from kDNA using oligonucleotide primers (S-366, S-367) to sequences which are not present in the D3 minicircle but presumably are present in the Lt26 sequence did not succeed. In addition, predicted Lt26 minicircle fragments were not detected using these oligonucleotide probes on MspI- or HaeIII-digested kDNA on Southern blots (data not shown). Finally, partial resequencing of two presumed Lt26 minicircle clones yielded the D3 sequence. We conclude that the previously reported Lt26 sequence was probably incorrect either as a result of sequencing errors or a cloning artifact and that the D3 sequence accurately represents this minicircle sequence class.

Multiple alignment of five *L. tarentolae* minicircle conserved regions

All five known *L. tarentolae* minicircles are organized similarly into a conserved region and a variable region. The extent of similarity of sequence in the conserved region can be seen by the multiple alignment of the conserved regions from the five known minicircle sequences in Fig. 2, which was obtained using the CLUSTAL program (22). The universal conserved CSB-3 12mer sequence, which represents an origin of replication for one strand, is embedded within a perfectly conserved 40 bp stretch. The length of the major region that is conserved among the five minicircle sequences varies from 167–176 bp. Another short conserved region (12 matches out of 18 bp) is located 30–44 bp downstream (Fig. 2). The variable regions show no sequence similarities except for the short motifs discussed below.

kDNA minicircles represented the first examples of bent DNA, which was originally identified by an abnormal mobility of certain restriction fragments in acrylamide gels (14,23,24). The sequence motif associated with the bend is phased runs of A residues at approximately 10 bp intervals (24–33). The B4, D3 and D12 minicircles each contain 5–6 sets of phased runs of A residues localized within and just outside the conserved region, as shown in Fig. 1. This localization is similar to that found previously in the Lt154 and Lt19 minicircles (14) and corresponds to the region of abnormal acrylamide mobility in those molecules and

presumably to the structural bend. We have not examined B4, D3 and D12 minicircle fragments for abnormal acrylamide mobility, but it is likely from the similarity of the motifs and the conservation of localization of the motifs within the molecules that there is a structural bend in this region.

Localization of gRNA genes on minicircles

Guide RNA genes had previously been mapped in the Lt154 minicircle sequence and the partial sequence of the D12 minicircle by Northern blot hybridization using specific oligonucleotide probes (2). The minicircle-encoded gRNAs are transcribed from the same strand and in approximately the same relative location in the variable region. This characteristic allowed for the prediction of gRNA locations for the D3, B4 and Lt19 minicircles. Northern analysis using oligonucleotide sequences indicated the existence of encoded gRNAs for all three minicircles (Fig. 3). The 5' ends of the gRNAs were determined by primer extension sequence analysis (Fig. 4). The distance of the 5' ends of the gRNAs from the conserved region (and the bend) is approximately 150 bp.

The 3' ends were determined for the D12 and Lt154 gRNAs both by analysis of the hybrids formed with edited mRNAs and directly by sequencing of chimeric gRNA/mRNA molecules. The 3' ends of the D3, B4, and Lt19 gRNAs were determined by sequencing unusual gRNA molecules obtained by PCR amplification of kinetoplast RNA using an oligo-[T] 3' primer and a gRNA-specific 5' primer. The products, which were possibly generated by self-priming of the oligo-[U] tail creating a template for the oligo-[T] 3' PCR primer, consisted of the gRNA with the oligo-[U] tail connected directly to a 3' oligo-[A] sequence. The D3 products showed two 3' terminal encoded nucleotides, indicated in Fig. 1B by arrows, indicating a slight 3' heterogeneity of either transcription termination or processing.

Each gRNA was shown to migrate in acrylamide as a family of bands running ahead of tRNA (data not shown). This type

Figure 2. Multiple alignment of conserved sequences of five minicircles from *L. tarentolae*. Clustal program (22). The CSB-3 sequence is boxed. Absolute matches are indicated by *, gaps introduced for alignment by —. The nucleotide numbering of the major conserved region refers to the Lt154 sequence.

Figure 3. Northern analysis of minicircle-encoded gRNAs. Formaldehyde-agarose gel of kinetoplast RNA. Lane 1, hybridization with cloned maxicircle pLt120 insert DNA, which shows the 9S and 12S rRNAs as size standards. Lane 2, D12 gRNA as a size standard. Lane 3, Lt19 gRNA. Lane 4, D3 gRNA. Lane 5, B4 gRNA. Lanes 6 and 7, hybridization with S-310 and S-311 probes from the D12 minicircle (See Fig. 1C), in an attempt to detect primary transcripts, additional gRNA transcripts or minicircle DNA contamination.

Figure 4. Sequencing of 5' ends of three minicircle gRNAs. The complement of each ladder sequence is written to the side of the autoradiogram. A. L19 5' end. B. B4 5' end. C. D3 5' end. See METHODS for primers used.

Figure 5. Alignment of five minicircle gRNA genes. The transcribed gRNA from each minicircle is shown (in lower case letters) and a portion of the 5' flanking minicircle sequence is also presented. The CSB-3 sequence is underlined and the intervening distances between this sequence and the 5' end of the gRNA is shown in bp for each minicircle (from : to :). The CCAAT and TAGTTGTA motifs are underlined.

of electrophoretic migration behavior, which can be attributed to heterogeneous 3' oligo-[U] tails as in the case of maxicircle-encoded gRNAs (3), had been demonstrated previously for Lt19 and D3 transcripts by two-dimensional acrylamide gel electrophoresis (34).

Minicircles probably encode single gRNAs in *L. tarentolae*

To address the question of the number of gRNA genes per minicircle molecule, two oligonucleotide probes (S-310 and S-311) for portions of the variable region of the D12 minicircle outside the known gRNA region were used for Northern analysis.

Figure 6. The complementarity of the D3 minicircle gRNA and a portion of the edited MURF4 mRNA. G-U base pairing is indicated by *, and standard base pairing by |. The gRNA nucleotides thought to be in the 3' anchor region are indicated by :s and guiding a and g nucleotides are written in lower case. The two alternative 3' terminal encoded A nucleotides in the gRNA sequence are underlined.

No hybridization signal was detected for either probe after extended exposure to X-ray film (Figure 3, Lanes 6, 7). This suggests that a single gRNA is encoded on this minicircle, unlike the situation in *T. brucei* in which three gRNA genes are located on a single minicircle (4). This evidence also eliminates the possibility that the minor high molecular weight band observed previously with an oligonucleotide probe for the D12 gRNA was due to either minicircle DNA contamination or to an unprocessed, full-length minicircle transcript (2). We have shown elsewhere that this band probably represents chimeric gRNA/mRNA molecules which are thought to be intermediates in editing (6).

Although we have not directly demonstrated that the four other minicircles also encode single gRNAs, this is likely to be the case due to the conservation of localization of the identified gRNA genes within the molecules relative to the conserved region.

Maxicircle-encoded gRNAs from *L. tarentolae* are primary transcripts, as shown previously by 5' capping experiments (3), and it is likely that this is also the case for these minicircle-encoded gRNAs, although this has not been demonstrated directly. In the case of the African trypanosomes, there is good evidence that minicircle-encoded gRNAs do represent primary transcripts (5).

Search for conserved transcriptional regulatory sequences

Both maxicircle-encoded and minicircle-encoded gRNAs (at least in *T. brucei*) are primary transcripts. However, in neither case are any transcriptional regulatory sequences known. Alignment of the five known *L. tarentolae* minicircle gRNA genes in relation to the 5' termini of the transcripts was performed in an effort to detect common sequence motifs potentially involved in the regulation of minicircle transcription (Fig. 5). A CCAAT motif is present at approximately -95 bp in three of the five minicircles, and may appear as CAAT in D3, and CCAAT in B4.

A second sequence element, CGATAGTTGTA, is absolutely conserved in the Lt154, and Lt19 minicircles at -36 bp. The B4 minicircle has TAGTTGTAC at -35 bp and the D3 minicircle has AGTTGTA at -40 bp. However, in the D12 minicircle, there is only a very degenerate similar motif at -36 but there is an AGTGGT motif at -58 bp.

The DNA bend, which is identified from the phased A motif to be localized at the edge of the conserved region approximately 150 bp from the gRNA 5' end, represents another possible minicircle element of regulatory significance.

The 5' termini of the gRNA transcripts do not exhibit the 5'-AYAYA consensus sequence reported for the minicircle gRNA transcripts from the African trypanosomes (4,5), nor are the 18mer inverted repeat sequences which enclose the gRNA genes (4,5,16) present.

There is no obvious transcription termination or processing signal at the 3' ends of the minicircle gRNA genes such as the UUU motif present at the 3' end of all maxicircle gRNA genes (4). Minicircle gRNAs may possess unique transcription termination signals, or may undergo 3' processing. However if 3' processing occurs, it must be rapid, as no precursors are visible on Northern blots (Fig. 3, lanes 6 and 7).

D3 gRNA could mediate editing of a portion of the MURF4 mRNA

A perfect complementarity between D3 gRNA and mature edited MURF4 mRNA (35) is shown in Figure 6. The 3' anchor with unedited mRNA is 6 bp, but could be as long as 15 bp with partially 3' edited mRNA. With this 3' anchor, the D3 gRNA would provide the information for 16 uridine additions and 2 uridine deletions from the MURF4 mRNA beginning at editing site 19. The B4, D12 and Lt19 gRNA sequences show no complementarity with any known edited mRNA sequence.

DISCUSSION

We have sequenced three additional minicircles from three different sequence classes from *L. tarentolae* kDNA. This brings the total minicircle sequence classes completely described in this species to five. From the complexity of restriction digestion patterns in gels and from T ladders of random clones (14) and the previously reported additional partial minicircle sequences (15), an approximate estimate of 10–20 total minicircle sequence classes has been made. The five known minicircle sequences each have a conserved region and a variable region in which little if any sequence similarity can be detected. The extent of micro sequence heterogeneity between minicircles within the same sequence class has not been examined in detail in *L. tarentolae*, but has been shown to be significant in *C. fasciculata* (36,37). Micro sequence heterogeneity within the gRNA coding region may result in changes in the editing patterns encoded by the gRNAs and significantly increase the gRNA genomic complexity, but this has not been examined. We have estimated that approximately 50 additional gRNAs are required for the six recently described pan-edited G-rich cryptogenes in the *Leishmania* maxicircle (38). Further work is required to address the question of the total minicircle-encoded gRNA complexity in *L. tarentolae*. Another possibility, by analogy with the apparent importation of tRNAs into the mitochondrion (34,39), is that some gRNAs are also encoded in nuclear DNA and imported.

The specific genetic function of three of the five minicircle-encoded gRNAs is known. The D12 gRNA encodes editing information for sites 1–8 of the COIII gene (2), the Lt154 gRNA a portion of the G6 editing information (38), and the D3 gRNA a portion of the MURF4 editing information. We assume, but have not yet proven, that the Lt19 and B4 gRNAs mediate editing of portions of the five G-rich cryptogenes whose edited transcripts have not yet been determined.

Two candidates for transcriptional regulatory sequences at –95 bp and –36 bp were detected in the 5' flanking sequences of the five identified minicircle gRNAs, but both motifs appear to be degenerate in some molecules, so the functional significance of these sequences is not certain. Another candidate for involvement in transcriptional regulation is the conserved structural bend in the molecule, which localized at approximately –150 bp. The conserved sequence elements described in the

African trypanosomes at the 5' end of minicircle-encoded gRNAs and the 18mer inverted repeats precisely flanking the gRNAs (4,5,16) are not present in the *L. tarentolae* minicircles. The question of the localization of the promoter sequences for minicircle gRNA transcripts in *L. tarentolae* remains open.

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