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Minicircle-encoded guide RNAs from *Crithidia fasciculata*

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ABSTRACT

Although the mitochondrial uridine insertion/deletion, guide RNA (gRNA)-mediated type of RNA editing has been described in *Crithidia fasciculata*, no evidence for the encoding of gRNAs in the kinetoplast minicircle DNA has been presented. There has also been a question as to the capacity of the minicircle DNA in this species to encode the required variety of gRNAs, because the kinetoplast DNA from the C1 strain has been reported as essentially containing a single minicircle sequence class. To address this problem, the genomic and mature edited sequences of the MURF4 and RPS12 cryptogenes were determined and a gRNA library was constructed from mitochondrial RNA. Five specific gRNAs were identified, two of which edit blocks within the MURF4 mRNA, and three of which edit blocks within the RPS12 mRNA. The genes for these gRNAs are all localized with identical polarity within one of the two variable regions of specific minicircle molecules, approximately 60 bp from the "bend" region. These minicircles were found to represent minor sequence classes representing approximately 2% of the minicircle DNA population in the network. The major minicircle sequence class also encodes a gRNA at the same relative genomic location, but the editing role of this gRNA was not determined. These results confirm that kinetoplast minicircle DNA molecules in this species encode gRNAs, as is the case in other trypanosomatids, and suggest that the copy number of specific minicircle sequence classes can vary dramatically without an overall effect on the RNA editing system.

Keywords: kinetoplast DNA; maxicircles; minicircles; MURF4; RNA editing; RPS12; trypanosomatid

INTRODUCTION

Guide RNAs (gRNAs) are short 3′-oligo-uridylylated RNA molecules that mediate the uridine-insertion/deletion RNA editing of kinetoplast maxicircle DNA transcripts in the mitochondria of trypanosomatid protozoa (Simpson et al., 1993). A few gRNAs are encoded in the maxicircle DNA, but the majority are encoded in the thousands of catenated minicircle DNA molecules (Pollard et al., 1990; Sturm & Simpson, 1990b, 1991; Pollard & Hajduk, 1991). Kinetoplast minicircle DNA molecules in different trypanosomatid species vary in size from 465 bp to 2.5 kb and are organized, depending on the species, into one, two, or four conserved regions and an equivalent number of variable regions (Simpson, 1987). The conserved regions are highly conserved in different minicircles within trypanosomatid species and short segments within these regions are conserved between species and between genera. One 12-mer sequence (CSB-III) (Ray, 1989) found in all trypanosomatids contains the origin of replication for one strand of DNA (Ntambi & Englund, 1985; Ntambi et al., 1986; Sheline & Ray, 1989). In *Leishmania tarentolae*, the 0.9-kb minicircles contain a single gRNA gene within a single variable region (Sturm & Simpson, 1991), and in *Trypanosoma brucei*, the 1-kb minicircles usually contain three gRNA genes situated between 18-mer inverted repeats within the single variable region (Corell et al., 1993). In *L. tarentolae* and *T. brucei*, the "bend" region, which is defined by a decrease in mobility of DNA fragments in acrylamide gels caused by phased stretches of adenosine residues, is situated adjacent to the conserved region (Marini et al., 1982, 1984; Kidane et al., 1984; Ntambi et al., 1984; Kitchin et al., 1986). No function has yet been ascribed to this region of bent DNA, which is present in the minicircle DNA from every trypanosomatid species, except possibly those from *T. cruzi* (Degrave et al., 1988).

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The 2.5-kb kinetoplast DNA (kDNA) minicircles of the insect trypanosomatid, *Crithidia fasciculata* are organized into two antipodal conserved regions and two variable regions (Birkenmeyer et al., 1985; Ray et al., 1986; Sugisaki & Ray, 1987). A single bend region is situated 90° from the conserved regions within one of the two variable regions.

Although RNA editing of maxicircle transcripts has been described in *C. fasciculata* (Van der Spek et al., 1988, 1990, 1991), no evidence for minicircle-encoded gRNA genes has yet been presented. In fact, a question has been raised as to the capacity of the minicircle DNA in the C1 strain to encode the required variety of gRNAs, because this DNA has been reported as consisting essentially of a single sequence class (Birkenmeyer et al., 1985).

To address the question of the role of the kinetoplast minicircle molecules in encoding gRNAs in the C1 strain of *C. fasciculata*, partially and fully edited RNAs from the MURF4 and RPS12 cryptogenes were cloned and sequenced, and a gRNA library was constructed. Five gRNAs were identified that mediate the editing of portions of the MURF4 and RPS12 mRNAs, and the genomic localization of the genes for these gRNAs was determined.

RESULTS

Comparison of the minicircle and maxicircle DNA components of the kinetoplast DNA from different strains of *C. fasciculata*

Kinetoplast DNA was isolated from the C1 strain of *C. fasciculata*, which has been used previously for analysis of kinetoplast DNA replication; the Steinert strain (Hoeijmakers & Borst, 1982; Hoeijmakers et al., 1982a, 1982b), which has been used previously for maxicircle sequence analysis and identification of maxicircle-encoded gRNAs (Sloof et al., 1985, 1987; Benne et al., 1986; Van der Spek et al., 1988, 1990, 1991; Arts et al., 1993); and three strains from the American Type Culture Collection (ATCC). *Hae* III and *Rsa* I restriction enzyme digestions of kinetoplast DNA from these strains were compared in acrylamide gradient gels (Fig. 1). Because more than 95% of the kDNA is minicircle DNA, this method is useful for analyzing the overall complexity of the minicircle DNA. A qualitative comparison of the acrylamide band profiles suggests the presence of two groups or schizodemes – one consisting of the C1 and the three ATCC strains, and the other of the Steinert strain. The DNA from the C1 strain was shown previously by cloning and sequencing to contain a single major minicircle sequence class; we have not analyzed the number of minicircle sequence classes in the Steinert strain.

To compare maxicircle sequences from the Steinert strain with the C1 strain, the normally highly polymorphic G1–G2 region (Simpson et al., 1987; Souza et al., 1992, 1993; Thiemann et al., 1994) was PCR-amplified from the latter and 234 nt of the G1 sequence was obtained. A comparison of this to the published G1 sequence from the Steinert strain showed only one extra C nucleotide and one missing C nucleotide in the C1 strain (data not shown). This and other sequence data (D.A. Maslov & L. Simpson, unpubl. results) suggests that the maxicircle genomes of the C1 and Steinert strains are essentially identical, implying that these strains are closely related, in spite of the minicircle DNA differences.

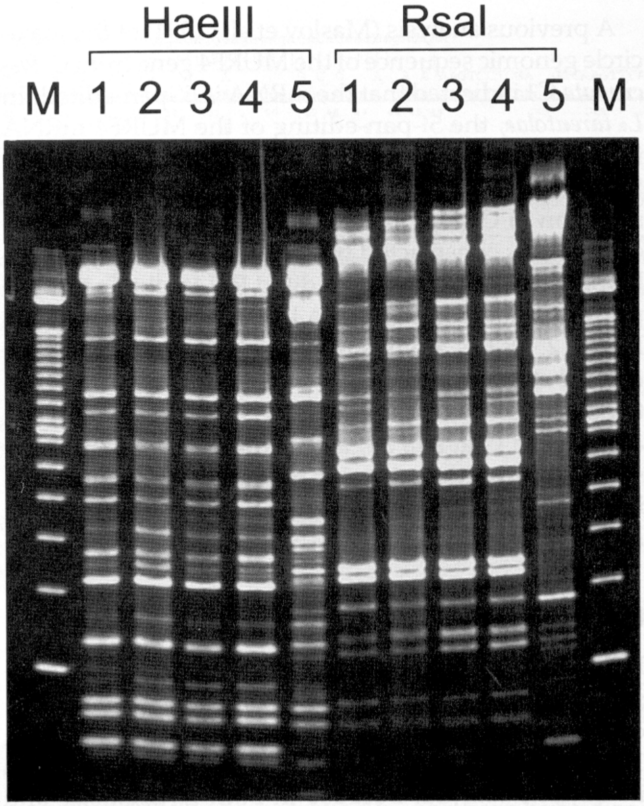

FIGURE 1. Comparison of digested kinetoplast DNA isolated from several strains of *C. fasciculata*. Approximately 4–5 μg DNA was digested with the indicated enzyme and the fragments separated in an acrylamide gradient gel (Morel et al., 1980). Lanes are the same for both enzymes: lane 1, C1 strain; lane 2, ATCC 11745; lane 3, ATCC 12857; lane 4, ATCC 12858; lane 5, Steinert strain. Lane M contains a 100-bp ladder size marker.

Editing of the MURF4 cryptogene

In order to investigate minicircle-encoded gRNAs in the C1 strain of *C. fasciculata*, it was necessary to first obtain the mature edited sequences of several pan-edited RNAs. The MURF4 and RPS12 cryptogenes were selected for this investigation, because both are pan-edited by multiple minicircle-encoded gRNAs in the phylogenetically closely related species, *L. tarentolae*, even in the gRNA-depleted UC strain of *L. tarentolae* (Thiemann et al., 1994).

A previous analysis (Maslov et al., 1994) of the maxicircle genomic sequence of the MURF4 gene from *C. fasciculata* C1 indicated that the mRNA is 5′-pan-edited. In *L. tarentolae*, the 5′-pan-editing of the MURF4 mRNA is mediated by six overlapping gRNAs (Maslov & Simpson, 1992). To analyze the precise MURF4 editing situation in *C. fasciculata*, a fully edited consensus sequence was constructed from an alignment of 15 overlapping clones of partially edited RNAs obtained from purified mitochondrial RNA by 5′RACE. As shown in Figure 2, 68 U's are inserted at 28 sites, and 3 U's are deleted at 3 sites. A possible methionine translation initiation codon is created by the insertion of a U between an A and a G at site 31, as occurs frequently in other species. The deduced amino acid sequence shows 81% identity with the MURF4 amino acid sequence from *L. tarentolae*.

Editing of the RPS12 cryptogene

In *L. tarentolae*, RPS12 (=G6) is one of the most extensively edited cryptogenes, the editing of which is mediated by eight overlapping gRNAs (Maslov et al., 1992). We examined the corresponding genomic region in *C. fasciculata* C1. Because in both *L. tarentolae* and *T. brucei* the G5–G6 region is surrounded by the unedited ND4 and ND5 genes (Simpson et al., 1987), the corresponding region was amplified using primers within the adjacent ND4 and ND5 genes. The product contained two G-rich sequences located on opposite strands, corresponding in location and polarity, but not in sequence, to the *L. tarentolae* G5 and G6 maxicircle sequences.

The 3′ mature edited sequence of transcripts from the G6 cryptogene was determined by the alignment of four sequences of partially edited G6 transcripts derived by RT-PCR (Sturm & Simpson, 1990a). The fully edited sequence was obtained by alignment of nine sequences derived by 5′ RACE, using a 3′-edited primer for cDNA synthesis (see the Materials and methods). In the mature edited RNA sequence, 136 U's are inserted at 59 sites, and 8 U's are deleted at 4 sites (Fig. 3). The number of U insertions is greater and the number of U deletions less than in the pan-edited RPS12 from *L. tarentolae* (Maslov et al., 1992). In addition, the unedited domain connection sequence I in the *L. tarentolae* sequence is edited in the *C. fasciculata* sequence, and domain connection sequence II is unedited, yielding two separate editing domains in *C. fasciculata* rather than three as in *L. tarentolae*.

The translated amino acid sequence shows 59% identity with that of *L. tarentolae* RPS12. The *C. fasciculata* sequence lacks the specific methionine putative translation initiation codon, which is conserved in *L. tarentolae* (Maslov et al., 1992), *T. brucei* (Read et al., 1992) and *Trypanoplasma borreli* (Maslov & Simpson, 1994), and has an AuA isoleucine codon at that position, but has an in-frame AUG codon several nucleotides upstream. The 3′ end of the *C. fasciculata* edited mRNA overlaps with the 5′ end of the ND5 mRNA by 8 nt (data not shown).

Identification of gRNAs for editing of MURF4 and RPS12

To identify gRNAs mediating the editing of the MURF4 and RPS12 mRNAs, a gRNA library was constructed from kinetoplast RNA (see the Materials and methods). Randomly selected clones were sequenced and the sequences compared with the edited MURF4 and RPS12 sequences. Two clones (cfg36 and cfg39) from this library were found to contain editing information for two blocks of MURF4 (Fig. 2). The absence of significant overlap between these editing blocks may imply the existence of another gRNA-mediated editing block located between these two.

```
                                                    cfg39
                             UUUAUAUCCCgUggaaaUgagagagggaUaUU–ACaaaaaUaaU–AUCCCU
                              || |  ...::::||||:|:|:|:::|:|:| ||||||||||| || | |               cfg36
                                                                           UUUaUCaaUaCggaCaUgaUaUgUgUaCCaag
                                                                           | :|||||:|.::||||:|||::|:||||||:
CfDNA TTTAATCAAATTTATTTAATTTTTAAAAGTAAA G     A          G GATTG     A  ATTACG AG  G A   G A  A G A A GG
CfRNA      UCAAAUUUAUUUAAUUUUUAAAAGUAAAuGuuuuuAuuuuuuuuuuGuGA*UGuuuuuAuuA*UACGuAGuuGuAuuuGuAuuAuGuAuAuGGuuu
                                       31  30       29   28 27    26   25 24   23  22 21 20 19 18 17161514  13
                                      M  F  L  F  F  F  C  D  V  F  I  I  R  S  C  I  C  I  M  Y  M  V  W

      CUaGaUCUUGGCACCGAG
      |||||||||::   |:|
CfDNA GA C AGAA   A G  G      A   AATA  G   AA    G GTACTGAG........
CfRNA GAuCuAGAAuuuAuGuuGuuuuuuAuuuAA*AuuGuuuAAuuuuGuGUACUGAG........
        12 11   10 9  8    7     6  5 4   3     2  1
        S  R  I  Y  V  V  F  Y  L  N  C  L  I  L  C  T  E   .......
```

FIGURE 2. Editing of *C. fasciculata* MURF4 mRNA and identification of two gRNAs. The genomic sequence (DNA) is shown above the mature edited sequence (RNA). The inserted U's are indicated by lower case u, the deleted U's by *. Editing sites are labeled 3′ to 5′ and the translated amino acid sequence shown below. Canonical base pairs between gRNA and mRNA are indicated by vertical bars, GU base pairs by colons, and AC base pairs by single dots. The sequence of the edited cDNA has been assigned GenBank accesion number U30222.

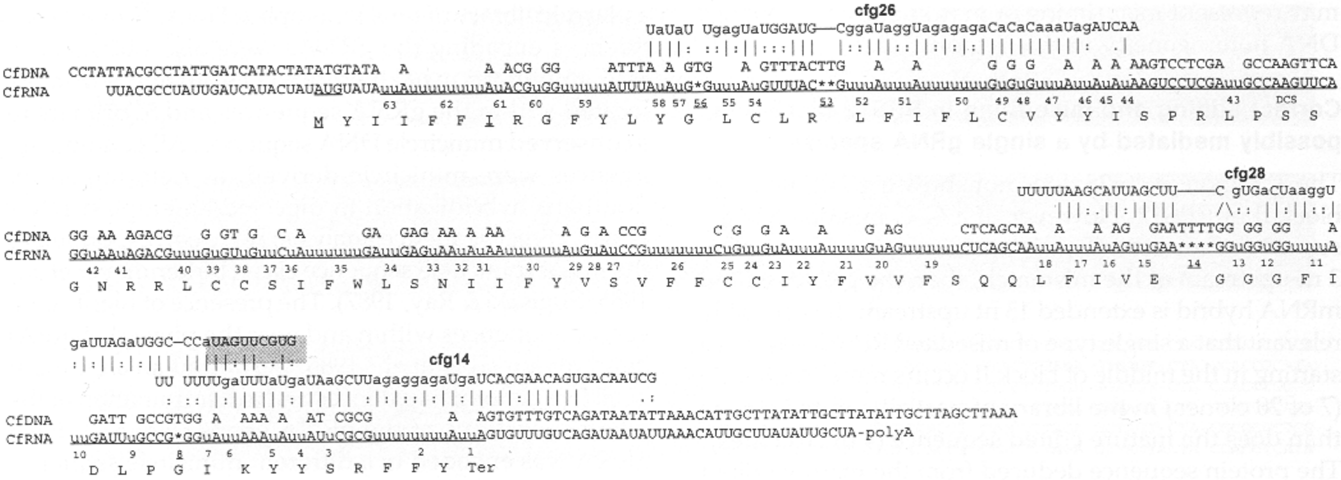

FIGURE 3. Editing of *C. fasciculata* RPS12 mRNA and identification of three gRNAs. See legend of Figure 2 for details. DCS, domain connection sequence. The anchor sequence is indicated by shading. The sequence of the edited cDNA has been assigned GenBank accession number U30223, and the genomic sequence, U30221.

The relative locations of these editing blocks within MURF4 correspond well to the locations of the cognate blocks in *L. tarentolae* (data not shown), and the total number of gRNAs involved is at least three, as was estimated previously (Maslov et al., 1994).

Three other clones (cfg14, cfg28, cfg26) were found to contain editing information for RPS12 (Fig. 3). The 3′ region of the cfg28 gRNA sequence, however, does not match well with the edited mRNA sequence without assuming a slippage during the editing process, as described below. There is a significant overlap of the cfg14-mediated block I sequence with the cfg28-mediated block II sequence, indicating that editing of block I is necessary for hybridization of block II gRNA.

The presence of all five gRNAs in steady-state kinetoplast RNA was confirmed by northern hybridization (data not shown), and the 5′ ends of the gRNAs were mapped by primer extension sequencing (Fig. 4). All gRNAs showed a single major 5′ end, except in the case of the cfg39 gRNA, which had a weak 5′ extension ladder beyond the presumed 5′ end, the sequence of which did not agree with the genomic sequence. This

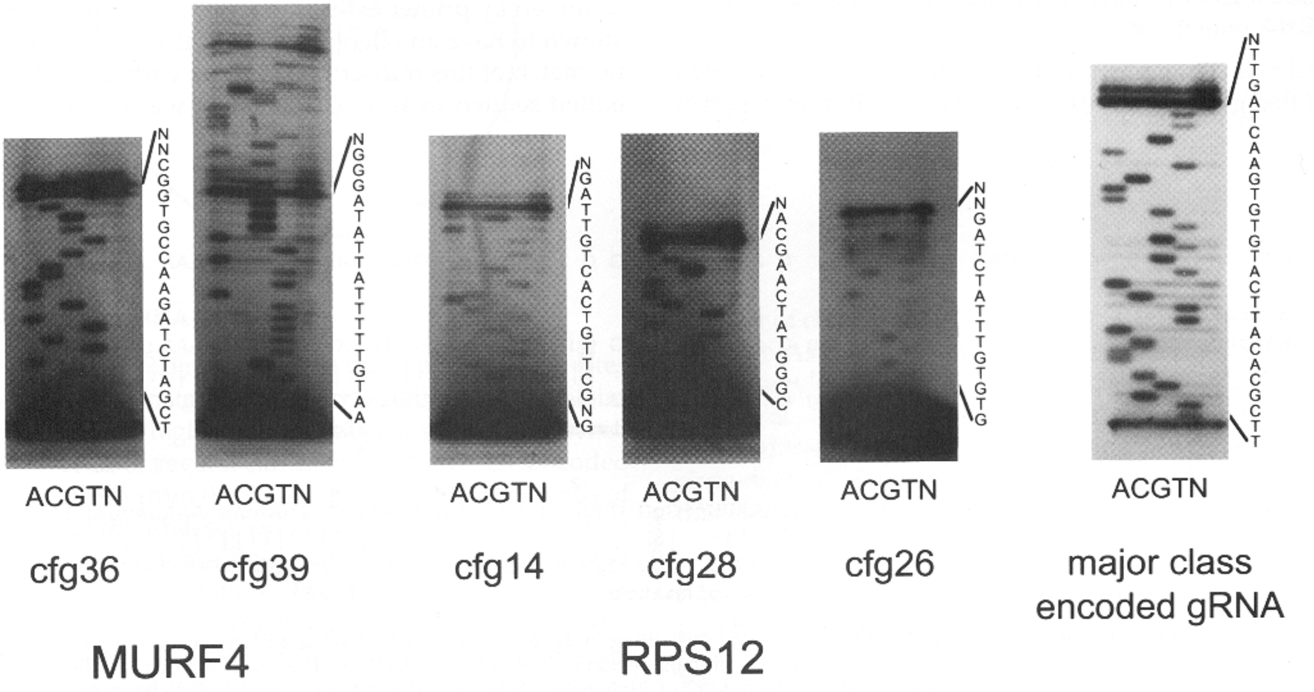

FIGURE 4. Primer extension sequencing of 5′ ends of six gRNAs.

may represent mispriming or may suggest a minicircle DNA heterogeneity in this region.

Correct editing and misediting in RPS12 block II is possibly mediated by a single gRNA species

The 3′ region of cfg28 does not show a good match to block II of RPS12. However, if a G-G mismatch is assumed as indicated (Fig. 5, position 1), and a slippage 3 nt upstream of the mismatch, then the gRNA-edited mRNA hybrid is extended 13 nt upstream. It is possibly relevant that a single type of misedited RPS12 sequence starting in the middle of block II occurs more frequently (7 of 20 clones) in the library of partially edited clones than does the mature edited sequence (4 of 20 clones). The protein sequence deduced from the mature edited sequence is identical to the *L. tarentolae* RPS12 sequence in this region, whereas the open reading frame from the misedited sequence does not show any similarity. The cfg28 gRNA sequence forms a duplex with this misedited mRNA sequence if a single U-U mismatch is allowed (Fig. 5, position 3). In this type of misediting pattern, the G-G mismatch occurring in correct editing is avoided by a U insertion in the mRNA (Fig. 5, position 2). This is a novel type of misediting pattern in which a single gRNA may guide both correct editing and misediting, depending on the precise mismatch that occurs. However, in order to properly analyze and confirm this phenomenon, a more complete library of edited RPS12 clones should be examined and the complete set of gRNAs obtained in order to eliminate the possibility that both of these represent misedited sequences.

Localization of five gRNA genes in kinetoplast DNA minicircles

Clones containing genomic sequences for the five identified gRNAs were selected by colony hybridization of a plasmid library of total kinetoplast DNA. Genomic sequences encoding the gRNAs were also obtained by PCR amplification of kinetoplast DNA using 3′ primers located within the gRNA sequences and 5′ primers to a conserved minicircle DNA sequence. All genomic sequences were minicircle-derived, as determined by Southern hybridization to digested kinetoplast DNA and by direct sequence analysis; none corresponded to the major minicircle sequence class (Birkenmeyer et al., 1985; Sugisaki & Ray, 1987). The presence of highly conserved sequences within and near the phased oligo[A] bend region (Ray et al., 1986) permitted an alignment of all five genomic sequences and a determination of the relative locations of the gRNA genes (Fig. 6A). Each gRNA was encoded in a different minicircle sequence class. All five gRNA genes were localized with the same polarity approximately 60 nt from the bend (Fig. 6B). No obvious conserved sequence motifs that may act as transcriptional promotors were present in the gRNA flanking regions.

An unassigned gRNA is encoded in the major class minicircle

The fact that five gRNAs are encoded at the same relative location on different minicircles prompted us to explore the possible existence of a gRNA transcribed from the corresponding region of the major sequence class minicircle. Although no transcripts from this region were found in the gRNA library, northern hybridization with an antisense oligomer probe showed that gRNA-sized transcripts are derived from this region (data not shown). The 5′ end of this transcript was determined by primer extension (Fig. 4), and the 3′ end shown to have an oligo[U] tail by 3′RACE. However, no match of this transcript sequence with any known edited sequences from *C. fasciculata* was found.

```
DNA        5'—CAG   CAA  A   A AG   G AATTTTG G G G  GG    A  GATT GCCGTGG A  AAA A  AT C—3'

correctly                             slippage
edited RNA 5'—CAG   CAAuuAuuuAuAGuu G AA****G GuG Gu GGuuuuAuuGAUUuGCCG*GGuAuuAAAuAuuAUuC—3'
                     ||  |:|:.||:|: | \/ \ /\/ :: 1| |:||::|:|:||:|:||| |||||:||::|:
                     UUUUUgagCaUUag C U   U  C gU Ga CUaaggUgaUUAGaUGGC CCaUAGUUCGUG
                                               mismatch           cfg28

misedited                                          2
RNA        5'—CAGuuuCAA  A   A AGuuuGuAAUU**GuG GuGuuGGuuuuAuuGAUUuGCCG*GGuAuuAAAuAuuAUuC—3'
                      |  |   | |::|:|||||:  |3: |:::||:||::|:|:||:|:||| |||||:||::|:
                      U  U   U UUgagCaUUAG  CUU CgUgaCUaaggUgaUUAGaUGGC CCaUAGUUCGUG
                                         mismatch                 cfg28
```

FIGURE 5. Possible mediation of correct editing and misediting in the RPS12 mRNA by a single gRNA. The presumed correctly edited RNA sequence is shown, with the slippage region indicated by highlighting. The GG mismatch is indicated by "1." The misedited RNA sequence observed in 7 of 20 clones is shown below with the U insertion that eliminates the GG mismatch indicated by "2." The remaining UU mismatch is indicated by "3."

A

```
M19266  CCGCCAAACC CCCTGCCAGG AGGCCTAAAA TTCCAGGCGA AAATCGCGAG GTTACTTTTT TGGAGCCC-G AAAAGTGCCC AAAATCAAGG -AAAAA--TG
cfm1    CCGCCAAACC CCCTGTCAGG AGGCCTAAAA TTCCAGGCGA AAATCGCGAG GTTACTTTTT TGGAGCCC-G AAAAA-CCCC AAAAAA-TGG CAAAAAAATC
cfm11   CCGCAGAAAC CCCTGCCAGG AGGCCCAAAA TTCCAGGCGA AAATCGCGAG GTTACTTTTT --GAGCCCCG AAAAA-CCCC AAAAAA-TGG CAAAAAA-T-
cfm21   CCGCCAA-CC CCCTGCCAGG AGGCCTAAAA TTCAAGGCGA AAATCGCGAG GTTACTTTTT T-GACGCCCG AAAAA-CCCC AAAA--TTGG CAAAAA--TT
cfm22b                   CCAGG AGGCCTAAAA TTCCAGCCCA AAACCGC-AA GTTACTTTT- CCGACCCCCA AAAAGTGCCC AAAA--CCAG CAAAAAAACG
cfm28b                   CCAGG AGGCCTAAAA TTCCAGGCGA AAATCGCGAA GTTACTTTTT TTGAGGC--G AAAAATGGCC AAAAAAATGG CAAAAAA-TG
                               *                                Bend region

M19266  GCCAAAAAA- TGCC--AAAA AACAGCGAAA ATACCCC-AA AAA--TCGAC -AAAAATGGT AAAAAATCCC ---------- --GCAATTTT ATT-GAATTT
cfm1    CCCAAAAAA- TGGC--AAAA AATGGCAAAA ACCCCC--AA AAAAATGCC- -AAAAATTCC CGATTTTCTC T--------- --G-AATTTT GGT-GAATTT
cfm11   GCCAAAAAAA TACCC-AAAA AATGGCAAAA CCACCCCGAA AA---TGCC- -AAAAATACC CGATTTTCCC C--------- --G-A-TTTT CCCCGAATTT
cfm21   GGCAAAAA-- TCGGGCAAAA AATGGCAAAA AAT-GGC-AA AAAA-TCCCC AAAAAATGGC -AATTTTCCC C-AATTTTCC CCG-AATTTT T-CTGAATTT
cfm22b  GGCAAAAAA- -CCCCCAAAA A-TCAAGGAA AATACCCCAA AAA--TAGC- AAAAAATAGC AAAAAATACC -GATTTTTAG GCG-AATTTT -CCCGAATTT
cfm28b  G-CAAAAA-- TGGCCCAAAA A-TGGCAAAA AC-ACCCCGA AAA--TGACC AAAAAACACC GAATTTTCCC ---------- --G-AATTTT CCC-GAATTT

M19266  TAAGGGAAAA A-TGGGC-AA AAAA-TGGCC AAAAACGCAC TCAAAAATCA AAATCTGAAC GTCTACGCAT CTGCTTTAGA GTCTGT-CTA CCAACCCGCA
cfm1    TTACCGAAAA AA-CCCC-GA AAAA-TGGCC AAAAATGACC T-AAAAATCA AAATCTGAAC GCCTACGCCT CTACTTTAGA GGGAGT-CTA CCACCGCATA
cfm11   TACCCCAAAA A-TAGGC-AA AAA-GTGGCC AAAAACGCAC T-AAAAATCA AAATCTGAAC GTCTACGCCT CTACTTTAGA GGATGT-CTA CCACCTGGGT
cfm21   TTACCCGAAA A-TAG-CCAA AAAA-TGGCC AAAAATGACC T-AAAAATCA ACTTCTGAAC GTCTACGCCT CTACTTTAGA GAGAGT-CTA CCACCGCATA
cfm22b  TCGGC-AAAA A-TACTCCAA AA--GTGGCC AAAAACGACG T-AAAAATCA ATTTTTGAAC GTCTACACCT CTGCTTTAGA GCTAGT-CTA CGCGCCCTCG
cfm28b  T-CGGCAAAA AATACCCCAA AA--GTGGCC AAAAACGACG T-AAAAATCA ATTTCTGAAC GTCTACGCCT CTACTTTAGA GC-AGTACTA CCACCTCATA

M19266  AGAGGTTAAA CGAGGCACC- -GTGTTAAAG CAAACTAGTT CACACATGAA TGTGCGAAAG ATAAAAGTAT GGCGATGCAG CGGCGTCTTG TCGTTGTATA
cfm1    AGGCGTTAAT GAATCGGT-- -ATGTTACTC TCTCCCTATA ATAAAAACAT TATAGGGAGA GAGTAAAGGT GCCCTATATT ACTGCTCCAC TAATATAGGC
cfm11   CA--GTTAAC AAGAGCCGCA -GCGAATCGA GGAGCCACGG TTCTAGATCG AACCATGTGT ATAGTACAGG CATAACTATT TAAATGCCGC AACTATACAA
cfm21   AAGCGTTAAA AGCACCAGGA CGTCTT-AAG CTAACAGTGA CAAGCACTAG TAGAGGAGAT TCGAATAGTA TTTAGTAATA CTATGAGCAT CGCACTCTTC
cfm22b  CTTCGCACAG TGTATGAGTC A--GTTTAAG TGCTTGATAC CCGGTAGATT AGTGGAATCA GTGCTTCGAT TACGAAGGAC TTATTCCACG GGCCGATTTG
cfm28b  AAGAGTTATC AATCTCTC-- -GTGTTCTAC TAACTAGATA AACACACAGA GAGATGGATA GGCGTAGGTA TGAGTTATAC CAAGCAATCT CGCATGCAAG
                                                gRNAs --------->

M19266  CTGTTCGAGC CTGGTACTTG ACCCTCCCCT TTCCCCGGCA AGTCTCCCCG GCCGTTCGCG TTCATGGCTC GGGCTAGCTT TGCTCGCCAT TCGTGGCTCC
cfm1    TCCG
cfm11   ATGGGACGAG CTAAACTGGC TCCCAATCGC CAGCATGACT AAGCAGGCAG ATCTCCCG
cfm21   TAAACCTGTA GAAGCCTTGG CGCTCACAGG TCGTGCTTGC CCTGTAAGCT TCAAGGACGC CGCGTGCCTG ATCAGTCTCG CTTGCACCAA GGAGATAAAT
cfm22b  CACGGATATC CCCTAATTTA TTCGGCACGA ATAAAACGGG TGTCCTTTGC AAACCGTCGG CCCTCTTAAC GGGAG
cfm28b  CCCTGACTCG CCTTTCTTCC CGTCAAGCCT CGTTCGTTCC GGCCGGGGCT CGATTGCTTT CGCCTATC
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B

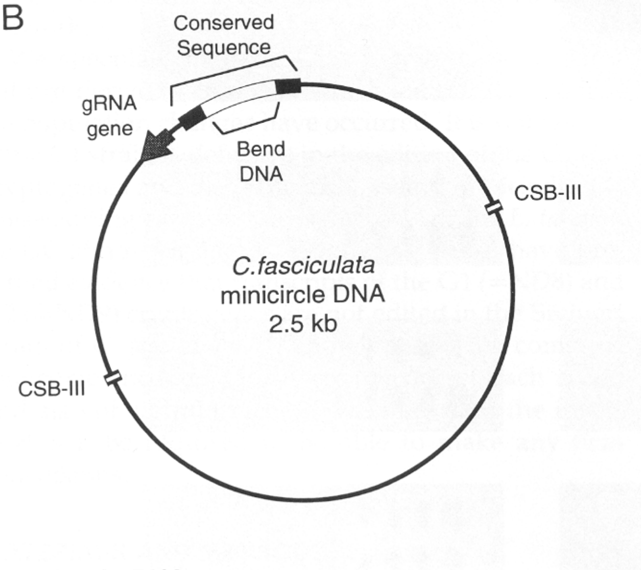

FIGURE 6. Localization of gRNA genes in specific minicircle DNA sequences. **A:** Sequences are aligned using the high level of conservation around the bend region. gRNA genes are shown by dark shading, the bend region by light shading. The region used for probing of conserved region in slot blot is shown by an asterisk (see the legend of Fig. 7). M19266, major minicircle sequence class (Sugisaki & Ray, 1987). The cfm1 sequence has been assigned GenBank accession number U30216; the cfm11 sequence, U30217; the cfm21 sequence, U30218; the cfm22b sequence, U30219; the cfm28b sequence, U30220. **B:** Diagrammatic representation of sequence organization of minicircle DNA in *Crithidia*. The conserved sequence around the bend used for alignment of the gRNA genes is indicated. CSB-III is a highly conserved 12-mer sequence involved in DNA replication (Ray, 1989).

3′ ends of gRNAs

Arts et al. (1993) reported that the 3′ uridylylation sites of maxicircle-encoded gRNAs from *C. fasciculata* (Steinert strain) are highly heterogeneous. We have analyzed the 3′ ends of several clones of the minicircle-encoded cfg26 gRNA from the C1 strain, and also the 3′ ends of clones of the unassigned gRNA encoded by the major minicircle sequence class. Nine cfg26 clones examined show two uridylylation sites that differ by a single nucleotide (data not shown). Seven of nine clones of the major gRNA class have a single uridylylation site, one has a site 2 nt upstream, and one has a site 3 nt upstream (data not shown). Although only a limited number of clones were analyzed, the 3′ termini of these two gRNAs did not show as large a variation as seen in the previously reported maxicircle-derived gRNAs (Arts et al., 1993). This may be due to a strain variation or to a difference in 3′ end processing between maxicircle-derived gRNAs and minicircle-derived gRNAs in this species.

Relative abundance of minor minicircle classes and the corresponding gRNAs

We analyzed the relative abundance of the minicircle sequence classes encoding the five identified gRNAs by differential hybridization, using a conserved region oligonucleotide probe and a probe unique to each minicircle class (Fig. 7). The results (Table 1) indicate that the five minicircle sequence classes together represent approximately 2% of the total minicircle DNA population and that the major sequence class represents greater than 90% of the minicircle population.

Qualitative northern blot analysis was performed to compare the steady-state abundance of the six identified minicircle-encoded gRNAs (data not shown). There is no correlation between the relative frequency of a specific minicircle sequence class and the relative abundance of the gRNA transcript. This conclusion, which is similar to that reached for minicircle-derived gRNAs in *L. tarentolae* (Maslov & Simpson, 1992), must, however, be confirmed by a quantitative analysis.

DISCUSSION

We show in this paper that the 2.5-kb kinetoplast minicircle molecules in *C. fasciculata* encode single gRNA genes at a defined location within one of the two vari-

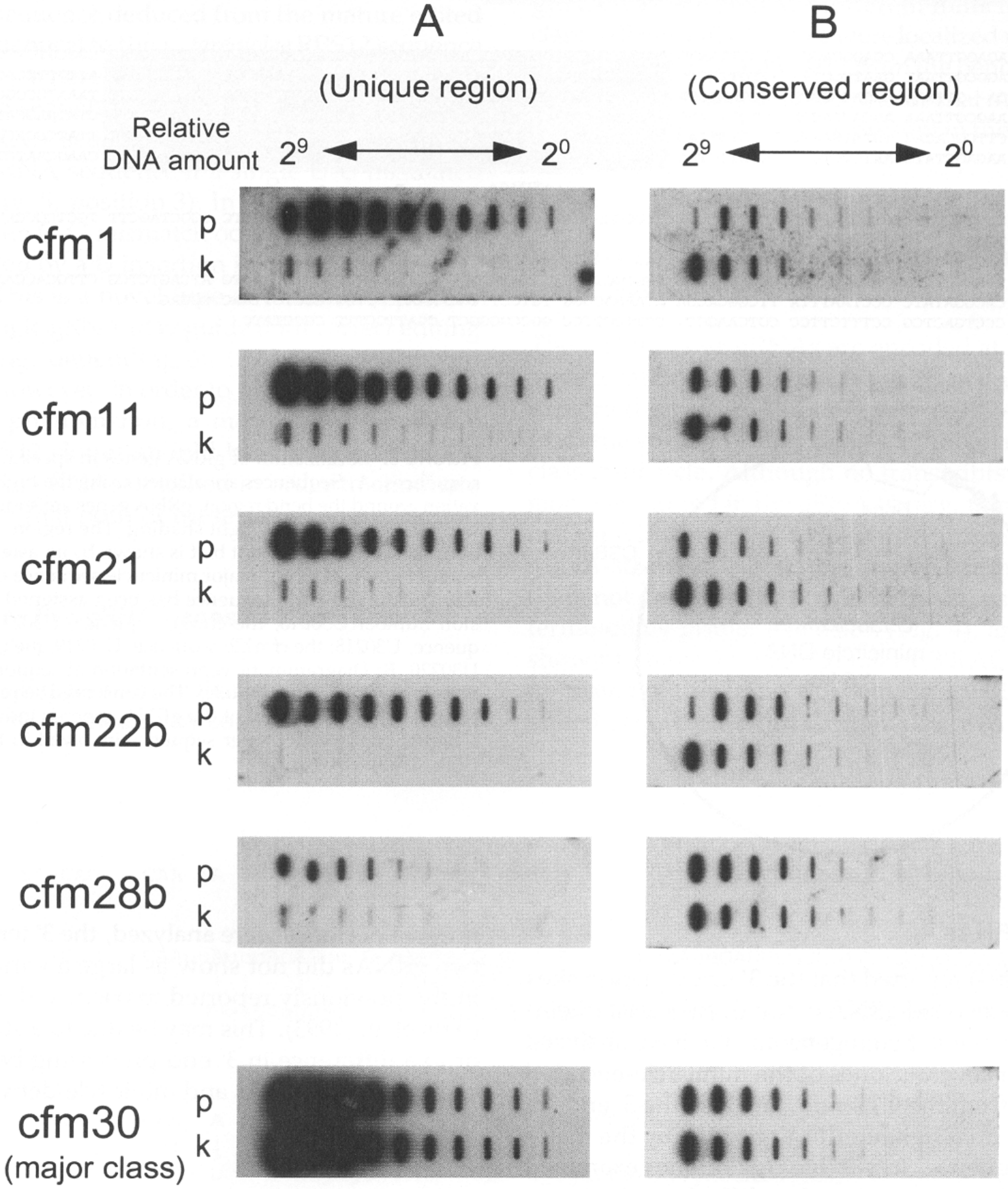

FIGURE 7. Copy number analysis of six minicircle classes including the single major class. **A,B:** Duplicate slot blots of the cloned minicircle fragment (p) and total kDNA (k) were hybridized with the (A) unique region or (B) conserved region-specific oligonucleotide. The sequence used for the conserved region-specific oligonucleotide is shown by an asterisk in Figure 6A. Each slot represents a doubling of the amount of DNA loaded, as indicated by 2^0–2^9. cfm30 represents a plasmid containing the gRNA+bend region of the M19266 major minicircle sequence class.

able regions. The localization of five randomly selected gRNA genes in five different minicircles in identical relative locations suggests strongly that each minicircle in the network encodes a single gRNA gene. We have not, however, analyzed the transcriptional activity of the remainder of the 2.5-kb minicircle, and the function of the second variable region is unknown.

The known editing patterns in *C. fasciculata* are very similar to those reported for *L. tarentolae* (Maslov et al., 1994), and therefore it is likely that an equivalent number of gRNAs are required for performing these editing events. In the case of the recently isolated LEM125 strain of *L. tarentolae*, 60 of an estimated 80 gRNAs have been cloned and identified, of which 43 are known to be minicircle-encoded (Thiemann et al., 1994). The old laboratory UC strain of *L. tarentolae* contains a smaller complement of minicircle-encoded gRNAs, and is defective in the editing of transcripts of the G1–G5 cryptogenes.

The C1 strain of *C. fasciculata* that was used in these studies contains a single major minicircle sequence class that comprises more than 90% of the minicircle DNA in the network (Birkenmeyer et al., 1985; Sugisaki & Ray, 1987). The five identified gRNA-encoding minor minicircle sequence classes represent approximately 2% of the 5,000 minicircle molecules in the network.

We speculate that the C1 strain may represent a culture-derived variant, in which substantial minicircle population changes have occurred. It is not known if the C1 strain is defective in the editing of the G1–G5 cryptogenes and lacks the gRNAs and minicircles for these editing cascades, as is the case for the *L. tarentolae* UC strain. However, Sloof et al. (1994) have presented evidence that transcripts of the G1 (=ND8) and G2 (=ND9) cryptogenes are not edited in the Steinert strain of *C. fasciculata*. A knowledge of the complete minicircle-encoded gRNA complexity of each strain and also of a strain recently isolated from the insect host will be required to be able to make any firm conclusions.

MATERIALS AND METHODS

Cell culture and kinetoplast DNA and RNA isolation

Strains 11745, 12857, and 12858 of *C. fasciculata* were obtained from the ATCC. Another strain was obtained from R. Benne and, because it was originally from M. Steinert (Kleisen et al., 1976; Hoeijmakers & Borst, 1982; Hoeijmakers et al., 1982b), we have labeled this the Steinert strain. The C1 strain is a clonal line that has been maintained in this laboratory in culture for more than 20 years (Simpson & Simpson, 1974). Cells were grown at 27 °C in brain–heart infusion medium (Difco) supplemented with 10 μg/mL hemin. Mitochondrial fractions were prepared from mid- to late-log phase cells by flotation in Renografin density gradients as described (Braly et al., 1974). Kinetoplast RNA was extracted from purified mitochondria (Simpson & Simpson, 1978), and kinetoplast DNA networks were purified from stationary cell cultures by sedimentation through a cesium chloride step gradient as described previously (Simpson, 1979).

TABLE 1. Relative abundance of minicircle sequence classes.

gRNA clone number	Cognate mRNA	Minicircle clone number	Relative abundance[a]
cfg36	MURF4(II)	cfm11	0.46%
cfg39	MURF4(III)	cfm1	0.39%
cfg14	RPS12(I)	cfm21	0.14%
cfg28	RPS12(II)	cfm22,22b	0.39%
cfg26	RPS12(VII)	cfm28a,28b	0.78%
	[b]	cfm30 (major class)	>90%

[a] All values represent the mean of two independent experiments.
[b] The editing role of this gRNA is not known.

Schizodeme analysis

Purified kinetoplast DNA was digested to completion with several restriction enzymes and the products separated by electrophoresis in acrylamide gradient gels (Morel et al., 1980). This qualitative method has been used previously to group *T. cruzi* strains into different schizodemes (Morel et al., 1980), which are defined as cells exhibiting different kinetoplast DNA restriction profiles.

Oligonucleotides

Oligonucleotide primers for PCR amplification, hybridization, and primer extension assays were synthesized by standard phosphoramidite methods and, in some experiments, purified by thin layer chromatography. The following oligonucleotides were used in this study.

Guide RNA library

S1405: CGCGGATCCAAAAAAAAAAAAAAC
S1406: CGCGGATCCAAAAAAAAAAAAAAG
S1407: CGCGGATCCAAAAAAAAAAAAAAT
S1408: GTTCCAGAATCGATAGTGAATTCGT

Cloning of fully edited MURF4 transcript

S1442: AAAAACATACATAAGCCAAT
S1443: CGCGGATCCAATAAATAAATAACCATCAA
S1483: ATACAAATACAACTACGTAT

5′ mapping of gRNAs and quantitative hybridization of minicircle classes

S1467: ATAGTTATGCCTGTACTATA
S1468: ATATAGGGCACCTTTACTCT
S1584: GCATCGCCATACTTTTATCT
S1605: TTAAATACTATTCGAATCTC
S1606: TAATCGAAGCACTGATTCCA
S1658: CTCATACCTACGCCTATCCA
S1803: CCAGGAGGCCTAAAATTCCAG

PCR amplification of G5–G6 region

S1514: CGCGGATCCTTTAATAT[AC]GATTT[CT]CC[AT]TTTATG
S1515: CGCGAATTCAATACAT[AG]TAATAAAATGCATAAAA

Cloning of partially and fully edited RPS12 transcripts

S1568: GCGAATTCGGGATTTAAGTGAGTTTAC
S1569: CCGGATCCTTTTTTTTTTTTTTTTTTT
S1608: ACCCGGCAAATCAATAAAAC
S1609: CGCGGATCCACCACCTTCAACTATAAAT

Cloning of gRNA-encoding region of minicircle DNA

S1467, S1468, S1605, S1606, S1658: shown above
S1696: CCCGGATCCAGGAGGCCTAAAATTCCA
S1697: CCCGAATTCCTCCCGTTAAGAGGGC
S1741: CCCGAATTCGGATTTCCGGGGTTGGTGTA
S1742: CCCGGATCCACACAGAGAGATGGATAG
S1784: CCCGAATTCGATAGGCGAAAGCAATCGA

PCR

PCR was performed in 50-μL reactions containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM or 5 mM $MgCl_2$, 0.1 mg/mL BSA, 200 μM of each dNTP, 0.5 μM of each primer, 2.5 units AmpliTaq, and appropriate template DNA, using the GeneAmp System PCR 9600 (Perkin Elmer). The PCR profile was 5 min at 94 °C, followed by 30 cycles at 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s.

5′ Mapping of gRNAs

A mixture of a 5′ end-labeled primer (3–5 pmol) complementary to a specific gRNA and kinetoplast RNA (5 μg) was denatured at 65 °C for 15 min and chilled on ice. Elongation–termination reactions were performed at the appropriate temperature for 30 min, using a 3:1 molar ratio of dideoxynucleotides and deoxynucleotides and AMV reverse transcriptase (Promega). The extension products were analyzed by electrophoresis on sequencing gels.

Cloning of edited MURF4 and RPS12 transcripts

Fully edited mRNAs of MURF4 were amplified with the 5′ AmpliFINDER RACE kit (Clontech). Partially edited RPS12 transcripts were amplified by RT-PCR (Sturm & Simpson, 1990a) using a 3′ oligo(dT) primer (S1569) and a 5′-unedited mRNA-specific primer (S1568). 3′-Edited primers (S1608 and S1609), based on the sequences of partially edited transcripts, were used to obtain a fully edited RPS12 transcript by 5′RACE PCR (Clontech).

Construction of a gRNA-cDNA library

gRNA-sized RNA (60–70 nt) isolated from a purified kinetoplast-mitochondrial fraction of *C. fasciculata* C1 was purified in a 10% polyacrylamide–8 M urea gel, and cDNA synthesis was primed by an oligo(dA) primer (S1405–S1407) complementary to the poly(U) tail of gRNAs. The cDNA was purified by polyacrylamide-urea gel electrophoresis, ligated to the anchor oligonucleotide included in the 5′AmpliFINDER kit (CLONTECH), and PCR amplified. PCR products were cloned into the pBluescript KS plasmid (Stratagene), and insert-containing clones chosen randomly were sequenced.

Cloning of gRNA-encoding genomic fragments

For the cloning of the kinetoplast DNA fragments encoding the cfg14, cfg28, cfg36, or cfg39 gRNAs, *Msp* I-digested kinetoplast DNA was separated in a 0.75% agarose gel, blotted, and the filters were hybridized with oligonucleotides specific for each of the gRNAs (S1605, S1606, S1467, or S1468, respectively). The DNA region showing a hybridization signal was gel-purified and ligated to the *Acc* I-digested pBluescript KS plasmid. The library was screened with the same oligonucleotide probes. Because the *Msp* I fragment that hybridizes to S1606 proved to contain a partial gRNA gene, the flanking region was PCR amplified, using a 3′ primer having the cloned *Msp* I fragment sequence (S1697) and a 5′ primer with the conserved bend region sequence (S1696). For cloning the kinetoplast DNA fragment encoding gRNA cfg26, the 3′ flanking region was PCR amplified with a 3′ primer complementary to the cfg26 sequence (S1742) and a 5′ primer with the conserved 12-mer sequence (S1741). Then the region containing the entire length of the cfg26 gene was amplified using a 3′ primer with the sequence of the previously amplified fragment (S1784) and a 5′ primer with the conserved bend region sequence (S1696).

Slot-blot hybridization of minicircle DNA

Total kinetoplast DNA or plasmids containing the cloned gRNA-encoding regions of minicircle DNA were digested by *Msp* I and serially diluted. These DNA dilutions were loaded onto Hybond N+ filters (Amersham) using the PR600 Slot-Blot apparatus (Hoefer Scientific Instruments). The blots were hybridized differentially with a conserved region oligonucleotide probe (S1803) or with unique region probes (S1467, S1468, S1584, S1605, S1606, S1658) at 45 °C for 16 h, and the blots were washed twice with 2× SSC, 0.1% SDS for 10 min each, and once with 1× SSC, 0.1% SDS for 15 min at 45 °C.

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