RNA (2000), *6*:121–135. Cambridge University Press. Printed in the USA.

Mitochondrial minicircles in the free-living bodonid *Bodo saltans* contain two gRNA gene cassettes and are not found in large networks

DANIËL BLOM,[1] ANNETT DE HAAN,[1] JANNY VAN DEN BURG,[1]
MARLENE VAN DEN BERG,[1] PAUL SLOOF,[1] MILAN JIRKU,[2]
JULIUS LUKES,[2] and ROB BENNE[1]

[1]Department of Biochemistry/AMC, University of Amsterdam, Academic Medical Centre,
1105 AZ, Amsterdam, The Netherlands
[2]Institute of Parasitology, Czech Academy of Sciences and Faculty of Biology,
University of South Bohemia, 37005, Ceské Budejovice, Czech Republic

ABSTRACT

In trypanosomatids, the majority of the guide (g) RNAs that provide the information for U-insertion/deletion RNA editing are encoded by minicircles that are catenated into large networks. In contrast, in the distantly related cryptobiid *Trypanoplasma borreli*, gRNA genes appear to reside in large 180-kb noncatenated DNA circles. To shed light on the evolutionary history and function of the minicircle network, we have analyzed minicircle organization in the free-living bodonid *Bodo saltans*, which is more closely related to trypanosomatids than *T. borreli*. We identified 1.4-kb circular DNAs as the *B. saltans* equivalent of minicircles via sequence analysis of 4 complete minicircles, 14 minicircle fragments, and 14 gRNAs. We show that each minicircle harbors two gRNA gene cassettes of opposite polarity residing in variable regions of about 200 nt in otherwise highly conserved molecules. In the conserved region, *B. saltans* minicircles contain a putative bent helix sequence and a degenerate dodecamer motif (CSB-3). Electron microscopy, sedimentation, and gel electrophoresis analyses showed no evidence for the existence of large minicircle networks in *B. saltans*, the large majority of the minicircles being present as circular and linear monomers (85–90%) with small amounts of catenated dimers and trimers. Our results provide the first example of a kinetoplastid species with noncatenated, gRNA gene-containing minicircles, which implies that the creation of minicircles and minicircle networks are separate evolutionary events.

Keywords: guide RNA; kinetoplastids; minicircles; mitochondrion; RNA editing

INTRODUCTION

In kinetoplastid mitochondria, the nucleotide sequence of mRNAs is posttranscriptionally revised by U-insertion/deletion editing under the direction of guide (g)RNAs (reviewed in Arts & Benne, 1996; Alfonzo et al., 1997; Stuart et al., 1997; Hajduk & Sabatini, 1998). A hallmark of this remarkable form of gene expression is the fact that the large majority of the gRNAs and the corresponding preedited RNAs are not encoded by the same DNA molecule. In members of the suborder Trypanosomatina (trypanosomatids), most of the gRNAs are encoded by minicircles that vary in size from 0.46 kb to 9.5 kb, depending on the species (Simpson, 1987; Jirku et al., 1995). The number of gRNA genes per minicircle varies, ranging from one in *Leishmania tarentolae* (Sturm & Simpson, 1991; Thiemann et al., 1994), *Crithidia fasciculata* (Yasuhira & Simpson, 1995), and *Phytomonas serpens* (Maslov et al., 1998), to (mostly) three in *Trypanosoma brucei* (Pollard et al., 1990; Riley et al., 1994) and four in *Trypanosoma cruzi* (Avila & Simpson, 1995) (reviewed in Simpson, 1997; for a complete list of all sequenced gRNA genes, see Hinz & Göringer, 1999). In addition, trypanosomatid minicircles contain sequences that are conserved in all species, such as a 12-nt CSB-3 sequence that contains the origin of replication for one of the DNA strands (Ray, 1989) and, with the possible exception of *T. cruzi* and *P. serpens*, a region of phased oligo (A) motifs, which induces bending of the DNA (Ntambi et al., 1984). Another characteristic feature of kinetoplast (k)DNA in trypanosomatids is the way it is organized into large networks consisting of thousands of catenated DNA molecules (Simpson, 1987; Borst, 1991; Shapiro & Englund, 1995), which in

---

Reprint requests to: Rob Benne, Department of Biochemistry/AMC, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands; e-mail: r.benne@amc.uva.nl.

addition to the minicircles also contain the 25–50 maxicircles that harbor the genes for (preedited) mRNAs and rRNAs. The function of the network is unknown, but it has been speculated that it may function as a primitive mitotic division device, ensuring ordered segregation of minicircles and maxicircles during kDNA replication (Borst, 1991).

Recently, the analysis of RNA editing and gRNA genes has been extended to species belonging to the suborder Bodonina, which comprises free-living bodonids as well as parasitic or commensalic cryptobiids. Phylogenetic studies have shown that bodonid and cryptobiid species represent early branches of the kinetoplastid tree (Fernandes et al., 1993; Lukes et al., 1994, 1997; Maslov et al., 1994; Wiemer et al., 1995; Blom et al., 1998a), which makes them interesting objects for the study of the evolutionary history of unusual processes such as RNA editing. So far, RNA editing has indeed been found in the cryptobiid *Trypanoplasma borreli* (Lukes et al., 1994; Maslov & Simpson, 1994) and the bodonid *Bodo saltans* (Blom et al., 1998a), suggesting an ancient origin for the editing process in the kinetoplastid lineage. gRNA gene organization has only been studied in *T. borreli*, in which the gRNA genes appeared to reside in tandem in noncatenated large 180-kb DNA circles (Yasuhira & Simpson, 1996). Given the position of *T. borreli* in phylogenetic trees, it has been speculated that these DNAs may represent evolutionary precursors of the minicircles (Yasuhira & Simpson, 1996; Simpson, 1997). Also, in other Bodonina species, large kDNA networks seem to be missing. In *Bodo caudatus* (Hajduk et al., 1986) and *Cryptobia helicis* (Lukes et al., 1998), noncatenated kDNA circles have been found of 10 and 12 kb, and 4.2 kb, respectively, that possess some of the features of trypanosomatid minicircles. The absence of a network appears to be reflected in the morphological appearance of Bodonina kDNA, which is found dispersed throughout the mitochondrial (mt) matrix (Vickerman, 1977; Brugerolle et al., 1979; Lukes et al., 1998). This arrangement has been termed "pankinetoplast" (Vickerman, 1977) to distinguish it from the typical disc-like kDNA structure of trypanosomatids. However, the conclusion that the noncatenated circular DNAs in *B. caudatus* and *C. helicis* mitochondria do indeed represent the equivalent of trypanosomatid minicircles cannot be drawn without the demonstration that they contain gRNA genes.

We are studying the mt genetic system of the free-living bodonid *B. saltans*. In a previous report, we described the analysis of a section of the *B. saltans* maxicircle and the features of edited RNAs (Blom et al., 1998a). During this work, we noticed the presence in *B. saltans* DNA of large amounts of noncatenated, 1.4-kb minicircle-like molecules, indicating that also in this species a large mt DNA network may be absent. In addition, *B. saltans* did not appear to possess a kDNA disc, but rather a more oval structure in a dilated region of the mitochondrion (Brooker, 1971; Blom et al., 1998b). In this article, we identify the 1.4-kb DNAs as the *B. saltans* equivalent of minicircles by showing that they contain gRNA genes and we demonstrate that the large majority of these circles are present as monomers. Therefore, we draw the conclusion that a kDNA network structure is not required for the ordered segregation of minicircles in *B. saltans*.

RESULTS

Cloning and sequencing of *B. saltans* minicircles

Previous electron microscopic (EM) and gel electrophoresis analyses of *B. saltans* DNA had revealed the presence of large numbers of minicircle-like DNAs of approximately 1.4 kb (Blom et al., 1998a). To further assess their identity, we cloned and sequenced a number of these molecules in procedures involving digestion of total DNA with either *Eco*RI or *Hin*dIII and ligation of the gel-excised linearized 1.4-kb DNAs into a cloning vector. From each procedure, two 1.4-kb inserts were sequenced. Comparison of their nucleotide sequences (Fig. 1A) showed that they contained regions of almost complete identity (nt 800–1150) interspersed with stretches of a more variable sequence, resulting in an overall identity of 63–74%. The conserved region proved to contain a sequence (GGGGTTGATATA) with 10 out of 12 matches to CSB-3 (GGGGTTGGTGTA), a conserved dodecamer motif present in all trypanosomatid minicircles, which is possibly involved in replication (Birkenmeyer et al., 1987; Ray, 1989). Adjacent to the conserved region, the sequences contained phased homopolymeric dA•dT tracts, similar to those found in trypanosomatid minicircles, in which they cause bending of the DNA and anomalous migration on polyacrylamide gels (Ntambi et al., 1984; Ray et al., 1986).

Next, we screened the sequences for the presence of gRNA genes, by looking for complementarity to the available edited mRNA sequences from *B. saltans* (Blom et al., 1998a) and other kinetoplastids. Indeed, we found two areas in pBMH06 and pBMH07 with the potential to form a perfect duplex of 46 and 49 nt, respectively, with parts of edited *B. saltans* ND5 mRNA, if G:U basepairing is allowed (Fig. 1B). Primer extension analysis with total *B. saltans* RNA and oligonucleotides complementary to the 3′ part of the encoded (putative) gRNAs, further showed the existence of RNAs with 5′ ends at the positions expected (Fig. 1B). From this, we concluded that these two 1.4-kb DNAs each contain a gRNA gene, completing their identification as the *B. saltans* equivalent of minicircles. The two ND5 gRNA genes were of opposite polarity and located in different regions that lack any obvious sequence identity to the corresponding regions of other *B. saltans* minicircles. Sections of other minicircles that aligned with these two gRNA genes (Fig. 1A) appeared to be the only two locations that harbor gRNA genes (see below) and they are referred to as gRNA gene cassettes I and II (see Fig. 1A,C). Both cassettes are preceded by a conserved 35-nt sequence element (inverted repeat, see Fig. 1A) and are flanked by stretches of 8–10 C residues, although the location of the C-stretch downstream of cassette I varied between minicircles. A map describing the features of a typical *B. saltans* minicircle is presented in Figure 1C.

Identification of additional minicircle-encoded gRNAs

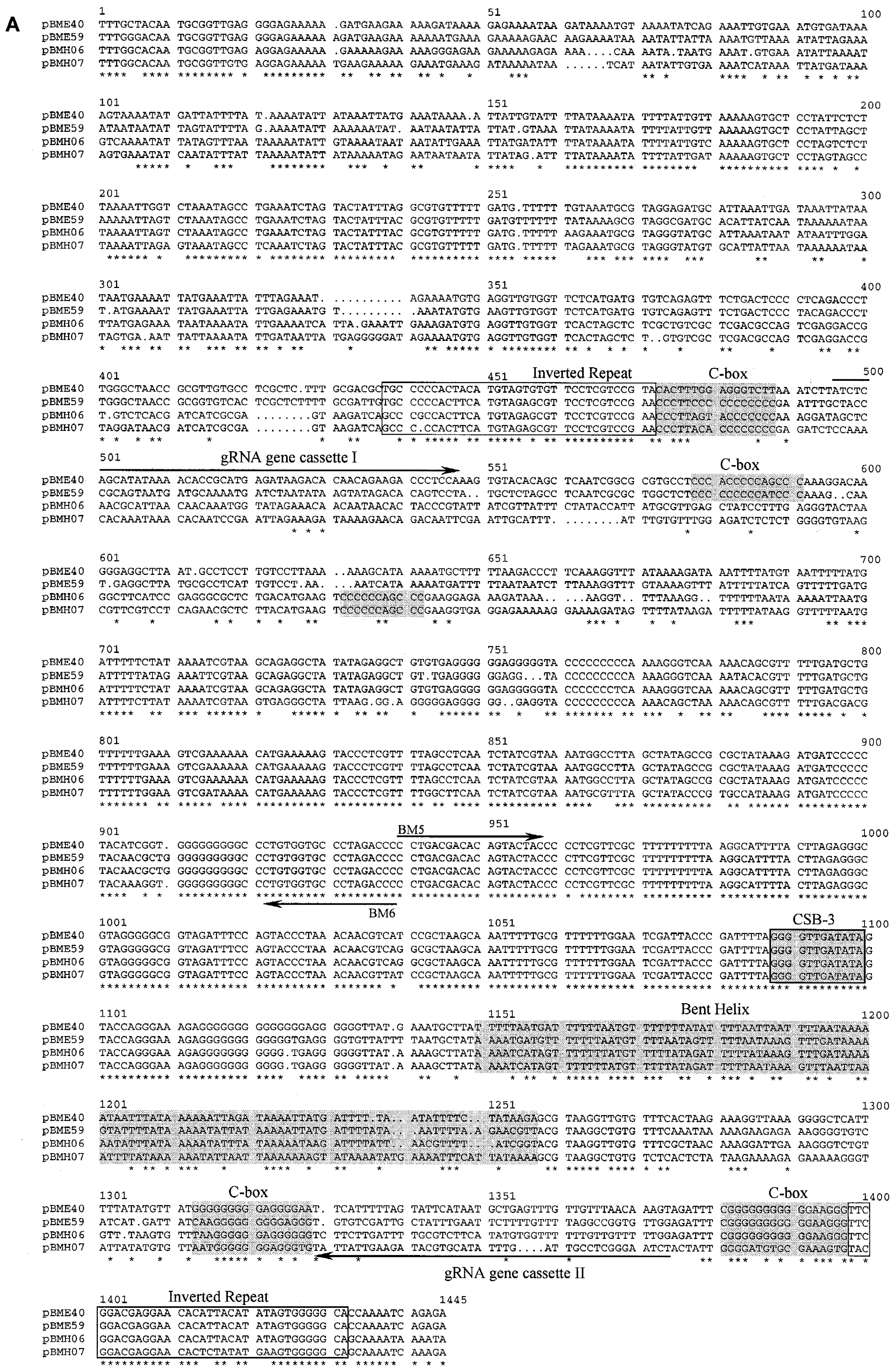
To further substantiate the analysis of the *B. saltans* minicircle gRNA gene organization, we constructed a gRNA-cDNA library from total *B. saltans* RNA, using the SMART-PCR cDNA synthesis kit. From this library, we obtained numerous insert sequences from which we selected 16 different candidate gRNAs using the following criteria: (1) presence of a 3′ U-tail, (2) insert length of 45–70 nt, (3) (partial) complementarity to edited kinetoplastid mRNAs (see Fig. 3) and/or lack of similarity to rRNA and mRNA sequences, and (4) overall purine richness, as guiding + anchoring sequences of most gRNAs contain 65–70% purines (Arts & Benne, 1996; Alfonzo et al., 1997; Stuart et al., 1997; Hajduk & Sabatini, 1998; see Fig. 1B). To determine whether these putative gRNAs are encoded by *B. saltans* minicircles, we designed a PCR approach using total *B. saltans* DNA and oligonucleotides specific for each of the candidate gRNAs in combination with either of two minicircle-specific oligonucleotides of opposite polarity and derived from abutted sections of the conserved region (BM5 and BM6, indicated in Fig. 1A,C). Of the 16 candidate gRNAs, 14 did indeed turn out be to minicircle encoded (Fig. 2A), as could be deduced from the appearance of a PCR product in this analysis. In 6 of the cases, the product was obtained with primer BM5, whereas the use of primer BM6 resulted in a band in 8 cases (Fig. 2B). The sizes of the PCR products generated were virtually identical (about 450 nt), irrespective of the specific primer combination used. This indicates that the genes for the 14 gRNAs that we cloned are found at only two locations on the minicircles and that these locations are equidistant from BM5 and BM6, in fact precisely corresponding to the location of the two identified ND5 gRNA genes (gRNA gene cassettes I and II, see Fig. 1A,C). Sequence analysis of each of the ~450 nt PCR products (data not shown, submitted to GenBank) and alignment of these sequences with each other and with the minicircles of Figure 1A, did indeed confirm that in all cases we had amplified either the part of the minicircle between gRNA cassette I and primer BM6 or that between gRNA cassette II and primer BM5. In some experiments, additional bands were produced during the PCR (e.g., with clones pBG 422 and 472 in the experiment shown in Fig. 2B). We have not further investigated the identity of the extra bands, in view of the fact that they were absent in other experiments and could be derived from PCR artifacts.

None of the 14 gRNAs possessed base pairing potential to sequenced, edited mRNAs from *B. saltans*, which is related to the fact that only a small part of the mt genetic system has been analyzed in this organism. In an attempt to identify the target preedited mRNAs nevertheless, we checked the ability of the gRNAs to base pair with mRNAs from *T. brucei*. The outcome of this analysis is shown in Figure 3. We found (near) perfect potential base pairing between a section of two *B. saltans* gRNAs and two domains of *T. brucei* ND7 mRNA (Fig. 3A,B). We also compared the *T. brucei* ND7 amino acid sequences encoded by the edited mRNA regions to those of *B. saltans* that can be inferred from the gRNA sequences. For these sections of the protein, this resulted in amino acid identities of 100% and 92%, respectively. Figure 3C–G lists all other alignments found in our analysis, in which the similarity between the inferred amino acid sequences from *T. brucei* and *B. saltans* is higher than 75%. Given this high degree of similarity, we conclude that we have indeed identified the cognate mRNAs of seven *B. saltans* gRNAs (see also Discussion).

B. saltans minicircles are not found in large networks

When total *B. saltans* DNA was analyzed by electron microscopy, no evidence could be found for the existence of a kDNA network in a number ($n = 6$) of different DNA preparations prepared without vortexing or other shearing. The *B. saltans* DNA did contain, however, very large chromosome-derived molecules (data not shown), demonstrating that the preparations contained intact, undegraded DNA. At high magnifications, a large number of 1.4-kb, relaxed DNA circles were visible, as determined from a comparison of their contour lengths with PM2 DNA, most likely representing the minicircles (Fig. 4A). Close inspection of the pictures also revealed the presence of catenated minicircle dimers and trimers (see inserts in Fig. 4A). In contrast, EM analysis of total DNA isolated from *C. fasciculata* using the same procedures readily revealed the typical trypanosomatid kDNA network structures (Fig. 4B).

The availability of cloned *B. saltans* minicircles allowed us to check for the possible presence of kDNA networks in total cellular DNA from *B. saltans* with techniques that separate DNA molecules on the basis of size. First, we compared the sedimentation pattern of *B. saltans* minicircles to that of minicircles from *C. fasciculata* on sucrose gradients, as visualized by a dot blot analysis of DNA extracted from gradient fractions (Fig. 5). It is clear that virtually all *B. saltans* minicircles



```
        1                                                      51                                                   100
pBME40  TTTGCTACAA TGCGGTTGAG GGGAGAAAAA .GATGAAGAA AAAGATAAAA GAGAAAATAA GATAAAATGT AAAATATCAG AAATTGTGAA ATGTGATAAA
pBME59  TTTGGGACAA TGCGGTTGAG GGGAGAAAAA AGATGAAGAA AAAAATGAAA GAAAAAGAAC AAGAAAATAA AATATTATTA AAATGTTAAA ATATTAGAAA
pBMH06  TTTGGCACAA TGCGGTTGAG AGGAGAAAAA .GAAAAAGAA AAAGGGAGAA GAAAAAGAGA AAA....CAA AATA.TAATG AAAT.GTGAA ATATTAAAAT
pBMH07  TTTGGCACAA TGCGGTTGTG AGGAGAAAAA TGAAGAAAAA GAAATGAAAG ATAAAAATAA ......TCAT AATATTGTGA AAATCATAAA TTATGATAAA
        ****  **** ******** *  *********  **  ** **  ** *   *     ***                ** *       ****  * **  * * * **

        101                                                    151                                                  200
pBME40  AGTAAAATAT GATTATTTTA T.AAAATATT ATAAATTATG AAATAAAA.A TTATTGTATT TTATAAAATA TTTTATTGTT AAAAAGTGCT CCTATTCTCT
pBME59  ATAATAATAT TAGTATTTTA G.AAAATATT AAAAAATAT. AATAATATTA TTAT.GTAAA TTATAAAATA TTTTATTGTT AAAAAGTGCT CCTATTAGCT
pBMH06  GTCAAAATAT TATAGTTTAA TAAAAATATT GTAAAATAAT AATATTGAAA TTATGATATT TTATAAAATA TTTTATTGTC AAAAAGTGCT CCTAGTCTCT
pBMH07  AGTGAAATAT CAATATTTAT TAAAAATATT ATAAAAATAG AATAATAATA TTATAG.ATT TTATAAAATA TTTTATTGAT AAAAAGTGCT CCTAGTAGCC
             *****  *   ***     ********   ***  *   **       * ****   *   ********** ********   ********** **** *  *

        201                                                    251                                                  300
pBME40  TAAAATTGGT CTAAATAGCC TGAAATCTAG TACTATTTAG GCGTGTTTTT GATG.TTTTT TGTAAATGCG TAGGAGATGC ATTAAATTGA TAAATTATAA
pBME59  AAAAATTAGT CTAAATAGCC TGAAATCTAG TACTATTTAC GCGTGTTTTT GATGTTTTTT TATAAAAGCG TAGGCGATGC ACATTATCAA TAAAAAATAA
pBMH06  TAAAATTAGT CTAAATAGCC TGAAATCTAG TACTATTTAC GCGTGTTTTT GATG.TTTTT AAGAAATGCG TAGGGTATGC ATTAAATAAT ATAATTTGGA
pBMH07  TAAAATTAGA GTAAATAGCC TCAAATCTAG TACTATTTAC GCGTGTTTTT GATG.TTTTT TAGAAATGCG TAGGGTATGT GCATTATTAA TAAAAAATAA
         ****** *   ********* * ******** *********  ********** **** *****    *** *** ****  ***       **      **     *

        301                                                    351                                                  400
pBME40  TAATGAAAAT TATGAAATTA TTTAGAAAT. .......... AGAAAATGTG AGGTTGTGGT TCTCATGATG TGTCAGAGTT TCTGACTCCC CTCAGACCCT
pBME59  T.ATGAAAAT TATGAAATTA TTGAGAAATG T......... .AAATATGTG AAGTTGTGGT TCTCATGATG TGTCAGAGTT TCTGACTCCC TACAGACCCT
pBMH06  TTATGAGAAA TAATAAAATA TTGAAAATCA TTA.GAAATT GAAAGATGTG AGGTTGTGGT TCACTAGCTC TCGCTGTCGC TCGACGCCAG TCGAGGACCG
pBMH07  TAGTGA.AAT TATTAAAATA TTGATAATTA TGAGGGGGAT AGAAAATGTG AGGTTGTGGT TCACTAGCTC T..GTGTCGC TCGACGCCAG TCGAGGACCG
        *  *** **  **  *** ** ** * **      *          ** ***** * ******** ** *  * *  *    *     **     *      **  **

        401                                                    451                                                  500
pBME40  TGGGCTAACC GCGTTGTGCC TCGCTC.TTT GCGACGCTGC CCCCACTACA TGTAGTGTGT TCCTCGTCCG TACACTTTGG AGGGTCTTAA ATCTTATCTC
pBME59  TGGGCTAACC GCGGTGTCAC TCGCTCTTTT GCGATTGTGC CCCCACTTCA TGTAGAGCGT TCCTCGTCCG AACCCTTCCC CCCCCCCCGA ATTTGCTACC
pBMH06  T.GTCTCACG ATCATCGCGA ........GT AAGATCAGCC CGCCACTTCA TGTAGAGCGT TCCTCGTCCG AACCCTTAGT ACCCCCCCAA AGGATAGCTC
pBMH07  TAGGATAACG ATCATCGCGA ........GT AAGATCAGCC C.CCACTTCA TGTAGAGCGT TCCTCGTCCG AACCCTTACA CCCCCCCCGA GATCTCCAAA
        * *  * **      *               *   **     * * ***** ** ***** * ** **********  ** ***         *   *

        501                                                    551                                                  600
pBME40  AGCATATAAA ACACCGCATG AGATAAGACA CAACAGAAGA CCCTCCAAAG TGTACACAGC TCAATCGGCG CGTGCCTCCC ACCCCCAGCC CAAAGGACAA
pBME59  CGCAGTAATG ATGCAAAATG ATCTAATATA AGTATAGACA CAGTCCTA.. TGCTCTAGCC TCAATCGCGC TGGCTCTCCC CCCCCCATCC CAAAG..CAA
pBMH06  AACGCATTAA CAACAAATGG TATAGAAACA ACAATAACAC TACCCGTATT ATCGTTATTT CTATACCATT ATGCGTTGAG CTATCCTTTG AGGGTACTAA
pBMH07  CACAAATAAA CACAATCCGA ATTAGAAAGA TAAAAGAACA GACAATTCGA ATTGCATTT. ........AT TTGTGTTTGG AGATCTCTCT GGGGTGTAAG
                                   * * *                                                   *        *              *

        601                                                    651                                                  700
pBME40  GGGAGGCTTA AT.GCCTCCT TGTCCTTAAA ..AAAGCATA AAAATGCTTT TTAAGACCCT TCAAAGGTTT ATAAAAGATA AATTTTATGT AATTTTTATG
pBME59  T.GAGGCTTA TGCGCCTCAT TGTCCT.AA. ...AATCATA AAAATGATTT TTAATAATCT TTAAAGGTTT GTAAAAGTTT ATTTTTATCA GTTTTTGATG
pBMH06  GGCTTCATCC GAGGGCGCTC TGACATGAAG TCCCCCCAGC CCGAAGGAGA AAGATAAA.. ...AAGGT.. TTTAAAGG.. TTTTTTAATA AAAATTAATG
pBMH07  CGTTCGTCCT CAGAACGCTC TTACATGAAG TCCCCCCAGC CCGAAGGTGA GGAGAAAAAG GAAAAGATAG TTTTATAAGA TTTTTATAAG GTTTTTAATG
          *    *        * *   *  *   **        **      * *                  *** *    *  *        ***          ** ***

        701                                                    751                                                  800
pBME40  ATTTTTCTAT AAAATCGTAA GCAGAGGCTA TATAGAGGCT GTGTGAGGGG GGAGGGGGTA CCCCCCCCCA AAAGGGTCAA AAACAGCGTT TTTGATGCTG
pBME59  ATTTTTATAG AAATTCGTAA GCAGAGGCTA TATAGAGGCT GT.TGAGGGG GGAGG...TA CCCCCCCCCA AAAGGGTCAA AATACACGTT TTTGATGCTG
pBMH06  ATTTTTCTAT AAAATCGTAA GCAGAGGCTA TATAGAGGCT GTGTGAGGGG GGAGGGGGTA CCCCCCCTCA AAAGGGTCAA AAACAGCGTT TTTGATGCTG
pBMH07  ATTTTCTTAT AAAATCGTAA GTGAGGGCTA TTAAG.GG.A GGGGGAGGGG GG..GAGGTA CCCCCCCCCA AAACAGCTAA AAACAGCGTT TTTGACGACG
        *****  **  *** ****** *    ***** *  ** **   *   ****** **  *   ** ******* ** ***  *  ** **    **** ***** *  *

        801                                                    851                                                  900
pBME40  TTTTTTGAAA GTCGAAAAAA CATGAAAAAG TACCCTCGTT TTAGCCTCAA TCTATCGTAA AATGGCCTTA GCTATAGCCG CGCTATAAAG ATGATCCCCC
pBME59  TTTTTTGAAA GTCGAAAAAA CATGAAAAAG TACCCTCGTT TTAGCCTCAA TCTATCGTAA AATGGCCTTA GCTATAGCCG CGCTATAAAG ATGATCCCCC
pBMH06  TTTTTTGAAA GTCGAAAAAA CATGAAAAAG TACCCTCGTT TTAGCCTCAA TCTATCGTAA AATGGCCTTA GCTATAGCCG CGCTATAAAG ATGATCCCCC
pBMH07  TTTTTTGGAA GTCGATAAAA CATGAAAAAG TACCCTCGTT TTGGCTTCAA TCTATCGTAA AATGCGTTTA GCTATACCCG TGCCATAAAG ATGATCCCCC
        ******* ** ***** **** ********** ********** ** ** **** ********** ****   *** ****** ***  ** ****** **********

        901                                                    951                                                  1000
pBME40  TACATCGGT. GGGGGGGGGC CCTGTGGTGC CCTAGACCCC CTGACGACAC AGTACTACCC CCTCGTTCGC TTTTTTTTTA AGGCATTTTA CTTAGAGGGC
pBME59  TACAACGCTG GGGGGGGGGC CCTGTGGTGC CCTAGACCCC CTGACGACAC AGTACTACCC CTTCGTTCGC TTTTTTTTTA AGGCATTTTA CTTAGAGGGC
pBMH06  TACAACGCTG GGGGGGGGGC CCTGTGGTGC CCTAGACCCC CTGACGACAC AGTACTACCC CCTCGTTCGC TTTTTTTTTA AGGCATTTTA CTTAGAGGGC
pBMH07  TACAAAGGT. GGGGGGGGGC CCTGTGGTGC CCTAGACCCC CTGACGACAC AGTACTACCC CCTCGTTCGC TTTTTTTTTA AGGCATTTTA CTTAGAGGGC
        ****  * *  ********** ********** ********** ********** ********** * ******** ********** ********** **********

        1001                                                   1051                                                 1100
pBME40  GTAGGGGGCG GTAGATTTCC AGTACCCTAA ACAACGTCAT CCGCTAAGCA AATTTTTGCG TTTTTTGGAA TCGATTACCC GATTTTAGGG GTTGATATAG
pBME59  GTAGGGGGCG GTAGATTTCC AGTACCCTAA ACAACGTCAG GCGCTAAGCA AATTTTTGCG TTTTTTGGAA TCGATTACCC GATTTTAGGG GTTGATATAG
pBMH06  GTAGGGGGCG GTAGATTTCC AGTACCCTAA ACAACGTCAG GCGCTAAGCA AATTTTTGCG TTTTTTGGAA TCGATTACCC GATTTTAGGG GTTGATATAG
pBMH07  GTAGGGGGCG GTAGATTTCC AGTACCCTAA ACAACGTTAT CCGCTAAGCA AATTTTTGCG TTTTTTGGAA TCGATTACCC GATTTTAGGG GTTGATATAG
        ********** ********** ********** ******* *   ********* ********** ********** ********** ********** **********

        1101                                                   1151                                                 1200
pBME40  TACCAGGGAA AGAGGGGGGG GGGGGGGAGG GGGGTTAT.G AAATGCTTAT TTTTAATGAT TTTTTAATGT TTTTTTATAT TTTAATTAAT TTTAATAAAA
pBME59  TACCAGGGAA AGAGGGGGGG GGGGGTGAGG GGTGTTATTT TAATGCTATA AAATGATGTT TTTTTAATGT TTTAATAGTT TTTAATAAAG TTTGATAAAA
pBMH06  TACCAGGGAA AGAGGGGGGG GGGG.TGAGG GGGGTTAT.A AAAGCTTATA AAATCATAGT TTTTTTATGT TTTTATAGAT TTTTATAAAG TTTGATAAAA
pBMH07  TACCAGGGAA AGAGGGGGGG GGGG.TGAGG GGGGTTAT.A AAAGCTTATA AAATCATAGT TTTTTTATGT TTTTATAGAT TTTTAATAAA GTTTAATTAA
        ********** ********** ****  **** ** *****    **   *       * **  * ***** **** ***  **  * *** *  **   ** *   **

        1201                                                   1251                                                 1300
pBME40  ATAATTTATA AAAAATTAGA TAAAATTATG ATTTT.TA.. ATATTTTC.. TATAAGAGCG TAAGGTTGTG TTTCACTAAG AAAGGTTAAA GGGGCTCATT
pBME59  GTATTTTATA AAAATATTAT AAAAATTATG ATTTTATA.. .AATTTTA.A GAACGTTACG TAAGGCTGTG TTTCAAATAA AAAGAAGAGA AAGGGGTGTC
pBMH06  AATATTTATA AAAATATTTA TAAAAATAAG ATTTTATT.. AACGTTTT.. .ATCGGTACG TAAGGTTGTG TTTCGCTAAC AAAGGATTGA AAGGGTCTGT
pBMH07  ATTTTATAAA AAATATTAAT TAAAAAAAGT ATAAAATATG AAAATTTCAT TATAAAAGCG TAAGGCTGTG TCTCACTCTA TAAGAAAAGA GAAAAAGGGT
            * ** * ***   *     ****  *   **    *        ***     *      ** ***** **** * **        ***     *

        1301                                                   1351                                                 1400
pBME40  TTTATATGTT ATGGGGGGGG GAGGGGAAT. TCATTTTTAG TATTCATAAT GCTGAGTTTG TTGTTTAACA AAGTAGATTT CGGGGGGGGG GGAAGGGTTC
pBME59  ATCAT.GATT ATCAAGGGGG GGGGAGGGT. GTGTCGATTG CTATTTGAAT TCTTTTGTTT TAGGCCGGTG TTGGAGATTT CGGGGGGGGG GGAAGGGTTC
pBMH06  GTT.TAAGTG TTTAAGGGGG GAGGGGGGTC TTCTTGATTT TGCGTCTTCA TATGTGGTTT TTTGTTGTTT TTGGAGATTT CGGGGGGGGG GGAAGGGTTC
pBMH07  ATTATATGTG TTAATGGGGG GGAGGGTGTA TTATTGAAGA TACGTGCATA TTTG....AT TGCCTCGGGA ATCTACTATT GGGGATGTGC GAAAGTGTAC
         *  *   *   *   ***** *  * *  *     *                    *        *                  **  ***  * *  * *** ** *

        1401                                    1445
pBME40  GGACGAGGAA CACATTACAT ATAGTGGGGG CACCAAAATC AGAGA
pBME59  GGACGAGGAA CACATTACAT ATAGTGGGGG CACCAAAATC AGAGA
pBMH06  GGACGAGGAA CACATTACAT ATAGTGGGGG CAGCAAAATA AAATA
pBMH07  GGACGAGGAA CACTCTATAT GAAGTGGGGG CAGCAAAATC AAAGA
        ********** ***  ** **   ******** ** ******  * * *
```

FIGURE 1. (*Legend on facing page.*)

B

B. saltans ND5 mRNA:
pBMH06 minicircle
cassette II sequence:

```
5' GuuuuuuuuuA-GUUuuuuuGAUUUuGUGUUUuuAUGuGUG---GuuuuuuGuuGUUuuuuuGuGuuuuG 3'
                |:||:|||||||||:||:||:||:||||   ||||||||||||||||::
             3' AGaagaaCUAAAaCGCAGAagUAUaCAC...CaaaaaaCaaCaaaaagg 5'
```

B. saltans ND5 mRNA:
pBMH07 minicircle
cassette I sequence:

```
5' uuGUUuuuAU---G-GuuGuuuGuuuuuuuAUuuuuuuA-AUUU-G-GAUU---GUGuuuAuuUGU-GUU--GGC--Guuuuuu 3'
                : :||||:||||:||||||:|||:|| |||: | ||||   |||||||||||| |||    |
             3' U.UaaCagaCaagaaaaUAgaaagaU.UAAG.C.CUAA...CACaaaUaaACA.CAA..ACC 5'
```

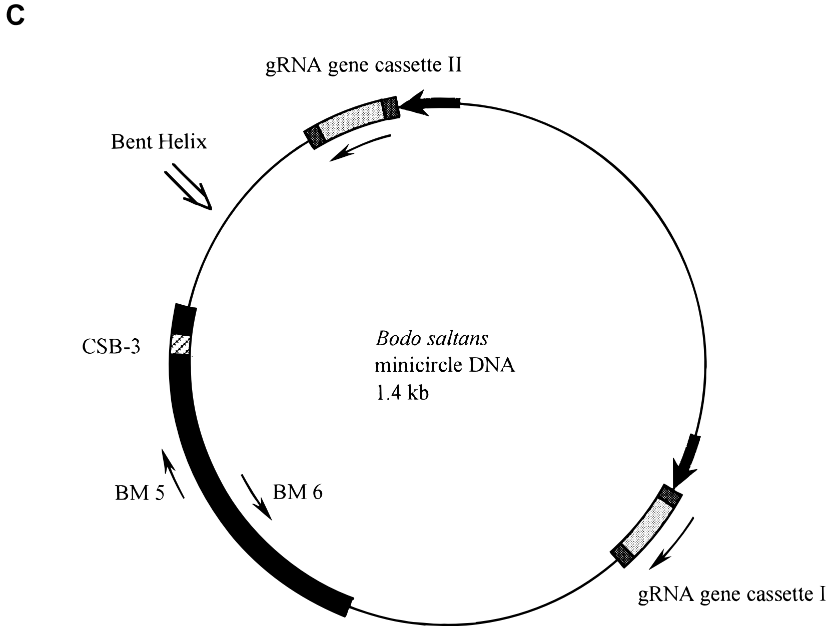


FIGURE 1. Sequence analysis of *B. saltans* minicircles. **A**: Comparison of the nucleotide sequences of four cloned minicircles. pBME40 and pBME59 are cloned *Eco*RI-digested minicircles, pBMH06 and pBMH07 cloned *Hind*III-digested minicircles. Gaps (.) have been introduced for optimal alignment of the sequences. gRNA gene cassettes I and II are indicated by long arrows, pointing in the direction of transcription. The C-boxes flanking the gRNA genes and the bent helix have been indicated by shading. Boxed regions represent 35-nt inverted repeats; the shaded, boxed region indicates the location of CSB-3. The positions of oligonucleotides BM5 and BM6, used for assignment of gRNAs to cassette I or II, have been indicated by short arrows. Asterisks indicate sequence identity. **B**: Identification of two putative ND5 gRNA genes. The possible base pairing is shown between two regions of *B. saltans* ND5 mRNA (top lines; nucleotides 1479–1545 in upper panel and 1542–1610 in lower panel) and sequences derived from cassettes II and I of *B. saltans* minicircle clones pBMH06 and pBMH07, respectively (bottom lines, 3′ → 5′, indicated by arrows in **A**. The 5′ ends of the minicircle sequences represent the 5′ ends of (g)RNAs encoded by the two genes, as determined by primer extension analysis. Conventional Watson–Crick base pairing is indicated by |, G:U basepairing by :. U residues inserted into the ND5 mRNA are shown by bold, lowercase Us, Us that are deleted from the mRNA are shown by −; dots have been introduced in the gRNA at those positions to align the sequences. **C**: Schematic representation of the *Bodo saltans* minicircle. gRNA-encoding cassettes are indicated by lightly shaded boxes and the C-boxes flanking these cassettes by darkly shaded boxes. The black box indicates the conserved region, in which the Conserved Sequence Block 3 (CSB-3) is indicated by hatching. Inverted repeats are depicted by black arrows; the bent helix is indicated by a white arrow; other symbols as in **A**.

sedimented at the top of the gradient (Fig. 5A), as expected for noncatenated minicircles. In contrast, about 40–50% of the *C. fasciculata* minicircles sedimented near the bottom of a parallel control gradient, at a position that corresponds to DNA with the size of the kDNA network (Fig. 5B). Next, we electrophoresed DNA from *B. saltans* and *C. fasciculata* in 0.8% agarose gels and compared the migration patterns of the minicircles on Southern blots (Fig. 6A). With *B. saltans* DNA, <1% of the hybridizing material was found in the slot of the lanes (Fig. 6A, lane 1), whereas the slots of the lanes in which *C. fasciculata* DNA were run contained 40–60% of the minicircles (Fig. 6A, lane 2). When this experiment was repeated with the Contour-clamped Homogeneous Electric Fields (CHEF) gel electrophoresis technique under conditions that allow large DNAs

A

gRNA clone	Sequence (5'->3')	gRNA gene cassette	Putative mRNA target
pBG 401	AAUUAUGCCACAAACAACAAGUGUUUCUGACAUAAUAUACAUUUUUUU	I	orphan
pBG 422	AGAGCAAUUACAAAACAAACCAUUAGCAGACAAAGAAACAGUAAUUUUUUUU	I	cox3
pBG 509	AGACCUAUAACACAACAUCCAAGAUGAUAGAACAAAACAACAGACAACUCCCCCCCUAAGAUUUUU	II	orphan
pBG 564	CCAACACCAACACAAAACCAAAGAAGAAAGACAAGUUACCAGAUUUUUU	II	orphan
pBG 572	CAACCGGUAUGAGACCUUCCAAGCAUUGAGCAGUUAAAGAAGACCCCCUAUAAAGUUUUUU	II	orphan
pBG 601	CAACCAAAACACAAAACACACAAUAGCAGACAAAGAUCACAACACCUGCUAUUUUUU	I	cox3
pBG 634	ACCUGCGGUGCAAAACAUCAUAAUCUAGAAGAAACAAACUAAUUUCUUCCAGAUAAUCUAUUAUGCUUUUUU	I	ND9
pBG 717 and 007	CGCUAUGCUCAUUACAACAAAUGCUGAUGUAAUCUAAUAACAAGAUUCCAUCUUUUUUUU	I	ND7
pBG 764	CACAAAAACAACAACAGCAUUGAAGCUAUCAACGAAAACAAUUUUUU	II	orphan
pBG 787	UCACGAACAACUAACAAACAGUAAGUCUGUGAUAUAAAGAACCAAUUUUUUU	I	ND3
pBG 846	CUACUCGCAAACAAACAUCCAAUGAGCAGAUGUACAAAAAAUCAGAUUUUUU	II	ND7
pBG 867	ACCUAUCGGCAAAAAACACAAGCAGUUGUACUAACAUCAAACAGAUUUUUU	II	orphan
pBG 896	CAGCAAAUCAAACAACAAAUCAAUUGAUAUGACAACAAAAAUCAUCUCAAUUUUUUU	I	orphan
pBG 928	AAUCAAUAAACAGACCGCAACAAGAAGAACUAAAUAGCAUCAACACUGCCAUUUAGUAGCGUUUUUU	I	cox3

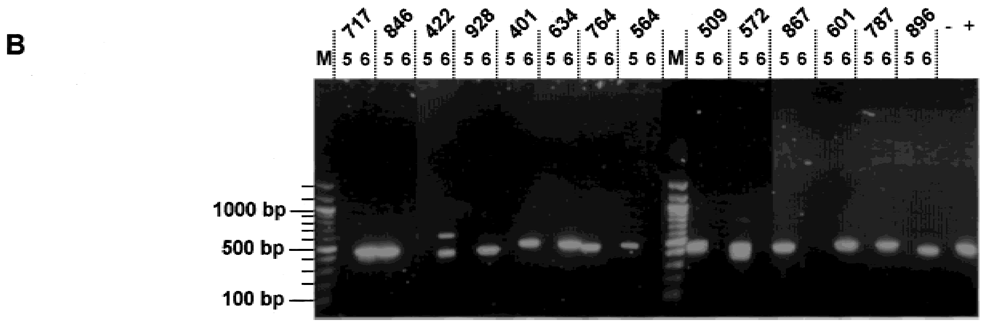


FIGURE 2. Identification of *B. saltans* gRNAs. **A**: The sequences are listed of 14 gRNAs from the gRNA-cDNA library that are minicircle-encoded (See **B**). The penultimate column indicates by which minicircle gRNA cassette each gRNA is encoded. Putative cognate mRNAs are also indicated, based on the potential to base pair with edited mRNA sequences from *T. brucei* (see also Fig. 3). **B**: Specific oligonucleotides derived from the gRNA sequences, as indicated above each pair of lanes by the number of the corresponding pBG clone (see **A**), were used in PCR reactions in combination with both BM5 (5-lanes) and BM6 (6-lanes) with total *B. saltans* DNA. Products were analyzed on 0.8% agarose gels, of which the ethidium-bromide stains are shown. The appearance of a product with BM5 (and not with BM6) demonstrates that the corresponding gRNA is encoded by cassette II. A product generated with BM6 (and not with BM5) indicates that the gRNA in question is encoded by cassette I. M: 100-nt ladder marker. + (Positive control): PCR primed with BM25, derived from the ND5 gRNA gene in cassette 1 (Fig. 1B), and BM6. − (Negative control): PCR primed with BM25 and BM5. Note that the picture is a compilation of several separate experiments.

of a size up to 500 kb to enter the gel, the result was essentially the same (Fig. 6B). This implies that the material present in the slots of the *C. fasciculata* DNA lanes represented minicircle networks of at least this size. Together, these results are consistent with the absence of large minicircle networks in *B. saltans*.

For further identification, each of the hybridizing bands of lane 1 of Figure 6A was isolated from gel and analyzed by EM. In the lower-molecular-mass region of the gel, we found two bands predominantly consisting of 1.4-kb DNA circles and one band containing 1.4-kb linear molecules. Digestion of the DNA with restriction enzymes prior to electrophoresis resulted in an increase in the intensity of the linear DNA band, at the expense of the two circular forms (results not shown). This clearly identified this band as the linear form of minicircles (indicated by l in Fig. 6). In analogy to the pattern of monomeric *C. fasciculata* minicircles (Kitchin et al., 1984; see Fig. 6A, lane 2) and plasmid DNA (Sambrook et al., 1989), we infer that the lower and the higher circular DNAs represented closed and nicked circular minicircles, respectively (indicated as c and n in Fig. 6). Depending on the DNA preparation, the circular forms of the *B. saltans* minicircles together comprised 55–75% of the total amount of minicircles, whereas about 15–30% was found in the linear form. We were unable to identify by EM the bands of hybridizing material migrating at higher-molecular-mass positions in the gel, most likely because of the low abundance of these molecules. However, from the position to which they migrated in the gels, we infer that they correspond to the catenanes of two and three

```
A                             L  P  Y  F  D  R  L  D  Y  V   S  V  C  C  N  E  H  L  L  S
T. brucei ND7 mRNA:       5' CUUCCGuAUUUCGACAGGUUAGAuuAuGuuA-GuGuuuGuuGuAAuGAGCAuuuGuuGuCu 3'    ID: 100%
                                       ||     :|||||||||:|:| |::|||||||||||||||||
pBG717 gRNA:         3' UUUUUUUUCUACCUUAGAACAAUAAUCUAAUGUAGU.CGUAAACAACAUUACUCGUAUCGC 5'
                                                L  D  Y  V   S  V  C  C  N  E  H

B                       R  L  R  C  A  D  F  L  H   I  C  L  L  D  V  C  L  R  G  F  L
T. brucei ND7 mRNA: 5' CGuuuGCGuuGuGCGGAuuuuuuGCA-UAUUUGuuuAuuGGAuGuuuGuuuGCGuGGuuuuuuA 3'    ID: 92%
                              :  :  | ||||||||| | :||:||:|:|||||||||||||||||| |
pBG846 gRNA:              3' UUUUUUAGACUAAAAAACAU.GUAGACGAGUAACCUACAAACAAACGCUCAUC 5'
                                       D  F  L  Y   I  C  L  L  D  V  C  L  R

C                        F  N  L  L  I  N  T  F  Y  L  F  V  S  G  L  F  V  N  F  F  V  L
T. brucei cox3 mRNA: 5' uuuAAuuuGuuGAuAAAuACAuuuuAUUUGuuuGuuAGuGGuuuAuuuGuuAAuuuuuuuGuuuuG 3'    ID: 78%
                                :  :| || |||  ||| |||||:||:||:|||||| |||  ||||| :|:|
pBG422 gRNA:                 3' UUUUUUUUAAUGACAAAGAAACAGACGAUUACCAAACAAAACAUUAACGAGA 5'
                                           T  V  S/F L  F  V  S  G  L  F  C  N  C  F

D                          G  V  F  V  F  L  S  L  P  A  I  V     I  V  Y  W  L  F  C
T. brucei cox3 mRNA:   5' GGuGuuuuuGuuuuuuuuAuCuuuACCuGCCAuuGu...uAuuGuGuAuuGGuuAuuuuGu 3'    ID: 73%
                                :  || || | ||||||  |||| |||||   | |||||| ||||||
pBG601 gRNA:         3' UUUUUAUCGUCCACAACACUAGAAACAGACGAUAACACACAAAACACAAAACCAAC   5'
                                             S  L  S  A  I  V  C  F  V  F  W  L

E                            C  L  Y  W  H  F  V  D  L  V  W  F  F  L  L  R  F  V  Y  F  D
T. brucei cox3 mRNA:     5' uGuuuAuAuuGACAuuuuGuuGAUUUAGuuuGAuuuuuuuuAuuGCGAuuuGuuuAuuuuGAu 3'    ID: 92%
                               :||:   :   :  ||||||| : :|||: ||:||:|| ||||| |:||||||||
pBG928  gRNA:        3' UUUUUGCGAUGAUUUACCGUCACAACUACGAUAAAUCAAGAAGAACAACGCCAGACAAAUAACUAA 5'
                                               V  D  V  V  C  F  F  L  L  R  F  V  Y

F                              L  E  R  E  C  F  D  L  F  G  F  Y  F  V  G  N  D  I  L  H  R  L
T. brucei ND9 mRNA:        5' uuGGAACGCGAAuGuuuUGAUUUGuuuGGuuuuUAuuuuGuuGGuAAuGAuAuuuuACAUCGuuuA 3'    ID: 92%
                                 : |   || |:|:| ||   : ||:|||| | |:|| | |:||||||| |||| ||:||:
pBG634 gRNA:         3 'UUUUUUUUCGUAUUAUCUAAUAGACCUUCUUUAAUCAAACAAAGAAGAUCUAUUACUACAAAACGUGGCGUCCA 5'
                                                          G  F  F  F  L  G  N  D  E  L  H  R

G                          N  S  R  L   V  F  Y  T  L  D  L  C  F  V  S  C  L  F  F
T. brucei ND3 mRNA:    5' AAuUCACGuuuG-GUGuuuuAuACAuuGGAuuuAUGuuuuGuuAGuUGuUUGuuuuuu 3'    ID: 86%
                               | : ||| || :||||||   ::||:|||:  |||||||||||||:||
pBG787  gRNA:            3' UUUUUUUAAC.CAAGAAAUAUAGUGUCUGAAUGACAAACAAUCAACAAGCACU   5'
                                    L   V  F  Y  I  V  D  L  L  F  V  S  C  L
```

FIGURE 3. Basepairing between *B. saltans* gRNAs and edited mRNAs from *T. brucei.* **A–G**: Potential duplexes between *B. saltans* gRNAs (bottom lines, 3′ → 5′) and sections of edited mRNAs from *T. brucei* (top lines, 5′ → 3′), as identified by the Bestfit program (GCG package). At the top and bottom of each panel, the (inferred) corresponding amino acid sequences of each species are shown. Identical amino acids (or amino acids resulting from conservative substitutions, F,Y; L,V,I; S,T; R,K; N,Q) are indicated in bold. The percentage of identity is indicated in each panel (ID). Conventional Watson–Crick base pairs are indicated by |, G:U basepairs by :. U-residues that are inserted into the mRNAs are shown by bold, lowercase Us; Us that are deleted from the mRNAs are shown by –. Dots have been introduced for optimal alignment.

interlocked minicircles (indicated in Fig. 6 as c2 and c3, respectively). The fact that these forms migrated as doublet bands is most probably related to the fact that different combinations of n and c forms can exist (e.g., n homodimers have a different mobility than n.c heterodimers). Quantification of the hybridization signals of the (putative) dimers and trimers revealed that they represented 5–10% and 1–5%, respectively, of the total amount of circular minicircles. In view of the fact that the CHEF gels were run in the absence of ethidium

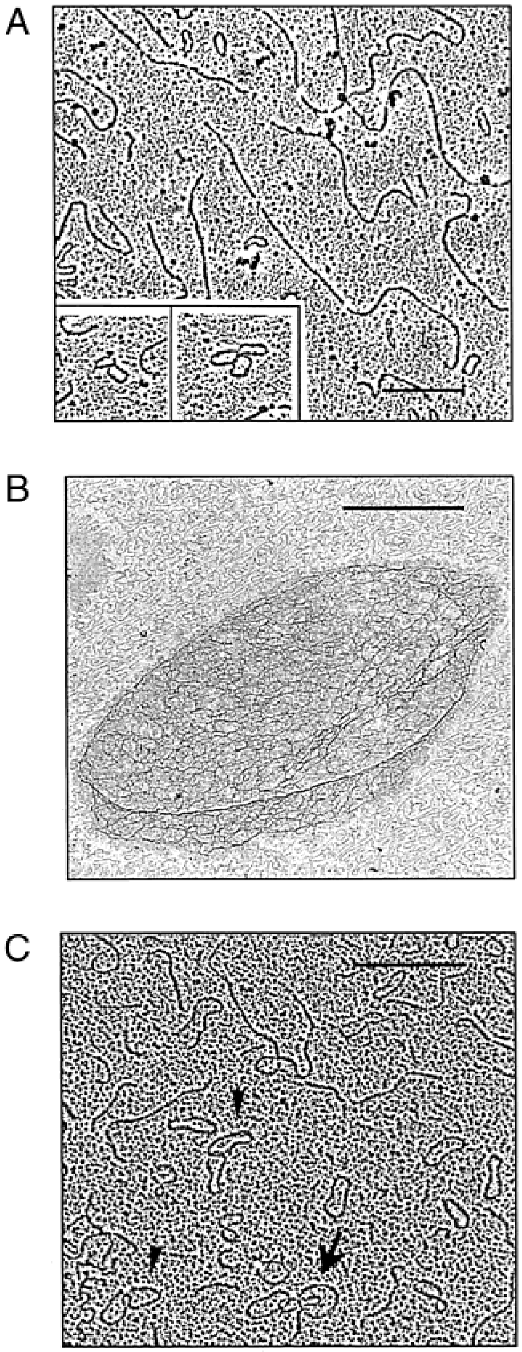


FIGURE 4. EM analysis. **A**: EM picture of total *B. saltans* DNA in which a number of 1.4-kb minicircles are visible. The inserts show a minicircle dimer and trimer. The bar represents 0.5 μm. **B**: EM picture of a kDNA network, as present in total *C. fasciculata* DNA. The bar represents 5 μm. **C**: EM picture of monomers, dimers (indicated with arrowheads) and trimers (bold arrow) of *B. saltans* minicircles, as present in upper band DNA from a CsCl-Hoechst gradient, which is enriched for minicircles (see text). The bar represents 0.5 μm.

bromide, the identification of the different hybridizing bands on Southern blots of these gels was less clear-cut. The assignments given in Figure 6B were inferred from EM analysis and a comparison between the hybridization patterns obtained with blots of CHEF gels and those of standard gels (cf. Figs. 6A and 6C).

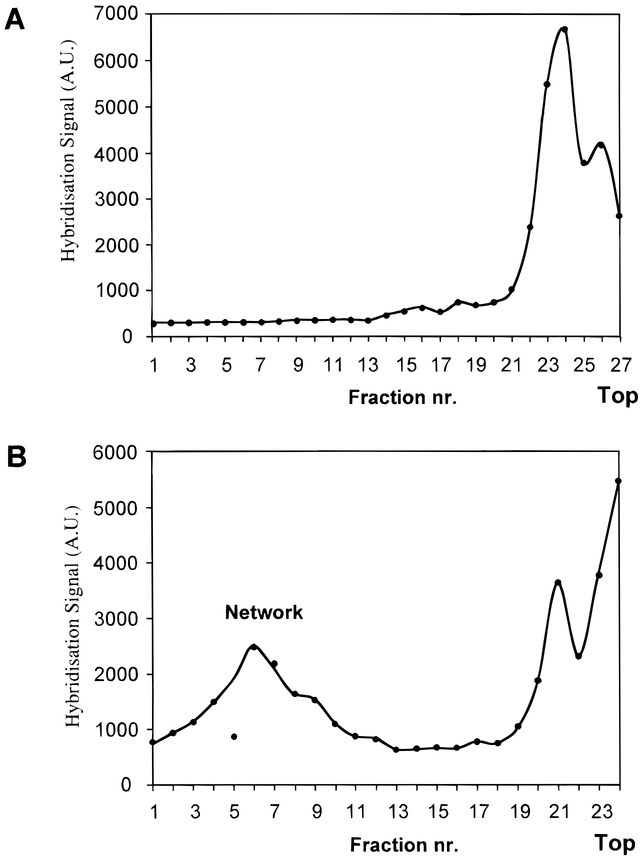


FIGURE 5. Sedimentation analysis of *B. saltans* and *C. fasciculata* minicircles. **A**: Sucrose gradient sedimentation of total *B. saltans* DNA was performed as described in Materials and Methods; DNA was isolated from each indicated fraction, spotted onto nitrocellulose filters, and hybridized with a *B. saltans* minicircle probe (pBME40). Radioactive signals were quantified with a phosphorimager and are indicated in Arbitrary Units (A.U.) on the vertical axis. Fraction 1 indicates the bottom fraction of the gradient, top fractions are on the right. **B**: Sucrose gradient sedimentation of total *C. fasciculata* DNA was performed as described in **A** for *B. saltans* DNA, with the difference that a *C. fasciculata* minicircle probe was used for hybridization (pDP312).

Finally, we performed CsCl gradient buoyant density centrifugation of *B. saltans* DNA in the presence of the A+T-binding dye Hoechst 33258, using a procedure designed to enrich for (AT-rich) mt DNA of other kinetoplastids (see, e.g., Simpson, 1979; Maslov & Simpson, 1994). After centrifugation, three discrete bands were detected (upper, middle, and lower bands in Fig. 6C) and DNA was isolated from each of them. As can be deduced from Figure 6C, in which similar amounts of DNA from the three bands were electrophoresed on an agarose gel and blotted, the upper band was highly enriched with minicircles (19-fold, see legend to Fig. 6C), containing more than 92% of the amount of minicircles present in total DNA. A similar enrichment was observed in experiments in which maxicircle probes were used (results not shown). We did

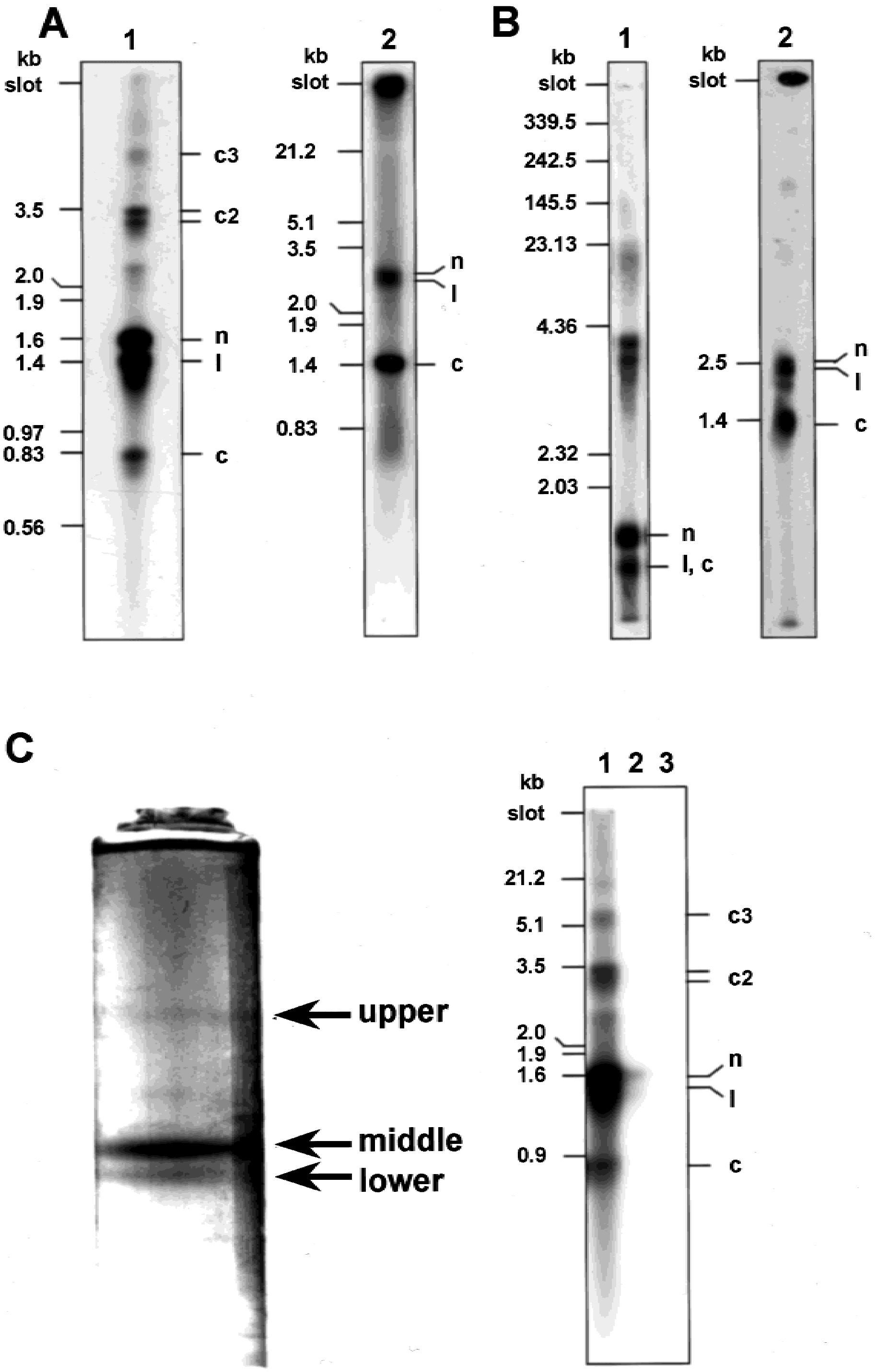


FIGURE 6. Gel electrophoresis and CsCl buoyant density gradient analyses. **A**: Total *B. saltans* DNA (lane 1) and total *C. fasciculata* DNA (lane 2) were electrophoresed on a 0.8% agarose gel (containing 0.5 μg ethidium bromide/mL) and blotted to nitrocellulose. The figure shows a picture of a Southern blot obtained after hybridization with a minicircle probe from *B. saltans* (lane 1) or *C. fasciculata* (lane 2). **B**: Same as in **A**, with the difference that samples were analyzed by Contour-clamped Homogeneous Electric Fields technique (CHEF), as described in Blom et al. (1998a). The gels were run in the absence of ethidium bromide; in some experiments, gels were stained with ethidium bromide after electrophoresis. **C**: Hoechst 33258-CsCl gradient of total *B. saltans* DNA (left panel). Three distinct DNA bands are indicated by arrows; DNA was isolated from these bands and analyzed by Southern blot hybridization with a *B. saltans* minicircle probe (right panel). Lanes 1–3 contain 250 ng of upper, middle, or lower band DNA, respectively. Indicated on the left side of each panel are sizes (in kb) of molecular-weight markers run in parallel lanes. Letters on the right side indicate the conformational state of the minicircles: c: closed circular; l: linear; n: nicked circular; c2: dimer; and c3: trimer.

not further analyze the contents of the middle and lower bands, but we infer that they contained the bulk of the nuclear DNA (and residual bacterial DNA) (Simpson, 1979; Maslov & Simpson, 1994). In line with the enrichment, EM pictures of the upper-band DNA showed the abundant presence of free 1.4-kb DNA circles, and a number of interlocked dimers and trimers in amounts of 13% and 3.0%, respectively, relative to the number of monomeric circles (see Fig. 4C). Most importantly, even in the material enriched with mt DNA, minicircle networks were conspicuously absent. This was confirmed by the migration patterns of the minicircles present in upper band DNA on agarose gels (Fig. 6C, lane 1), which were virtually identical to those obtained with total DNA (Fig. 6A), including the absence of hybridizing material in the slot.

DISCUSSION

Identification and characterization of *B. saltans* minicircles and gRNAs

In this article, we have characterized the minicircle component of mt DNA of the free-living bodonid *B. saltans*. There can be little doubt that the 1.4-kb molecules that we analyzed represent the *B. saltans* equivalent of minicircles, as they harbor two gRNA genes and possess other minicircle-like features, such as a conserved region that includes a (degenerate) CSB-3 sequence, and a bent helix DNA element (Fig. 1; see Ntambi et al., 1984; Ray, 1989; Shapiro & Englund, 1995; Simpson, 1997). The identification of the gRNA gene cassettes proved to be a multi-step process. First, we noted the potential of two almost diametrically opposed sequences on two different 1.4-kb DNA circles to form long duplexes (46 nt and 49 nt, respectively, including G:U pairs) with sections of edited ND5 mRNA from *B. saltans* (Fig. 1). In the next phase of this work, we showed that these sequences were indeed each transcribed into an RNA with a 5′ end at the expected position and we demonstrated that 14 45–70 nt, U-tail-possessing (putative) gRNAs were encoded at either one of these positions on different circles (Fig. 2).

The conserved region comprised a relatively large section of the *B. saltans* minicircles (nt 800–1140, see Fig. 1A), substantially larger than the conserved part of minicircles from most trypanosomatid species. Outside the fully conserved ($>$95%) region, there were other sections that are also relatively well conserved (see Fig. 1), leading to an overall conservation of 63–74%. In fact, the two gRNA gene cassettes that we have found resided in the only two sections that showed completely variable sequences. Together with the experimental evidence that localized the 16 identified gRNA genes to either one of the gRNA gene cassettes (Figs. 1 and 2), this strongly argues against the possibility of additional gRNA gene cassettes in *B. saltans* minicircles. Nevertheless, the present data do not completely rule out the existence of minor minicircle-sequence classes with different gRNA gene contents (Riley et al., 1994). Interestingly, both gRNA gene cassettes proved to be flanked by C-rich sequences and preceded by a 35-nt conserved element, which (due to the opposite polarity of the two cassettes) manifested itself as an inverted repeat in the minicircle sequences of Figure 1A. The role of these elements remains to be established, but their position and orientation relative to the gRNA genes suggests that they are involved in gRNA transcription or processing.

The identification of the cognate mRNAs of the 14 gRNAs extracted from the gRNA-cDNA library was less straightforward than that of the two ND5 gRNAs of Figure 1B, because none turned out to be complementary to the available *B. saltans* edited mRNA sequences. Therefore, we screened for complementarity with mt mRNAs from *T. brucei*, for which a complete set of sequences is available (see Arts & Benne, 1996; Alfonzo et al., 1997; Stuart et al., 1997; Hajduk & Sabatini, 1998). In addition, of all trypanosomatids that have been intensively studied, *T. brucei* is the closest relative of *B. saltans* (Blom et al., 1998a) resulting in nucleotide identities of the mt RNAs of $>$70%. For seven of the *B. saltans* gRNAs, this analysis revealed the potential to form a specific duplex of considerable length (35–45 nt, including G:U base pairs) to sections of edited mRNAs from *T. brucei* (ND7, cox3, ND9, and ND3 mRNAs), as outlined in Figure 3. This is underlined by a high similarity between the corresponding amino acid sequences, as inferred from the gRNA and mRNA sequences: 73–100%, for a stretch of 11–14 amino acids. This degree of similarity strongly suggests that we have identified the cognate mRNAs for these gRNAs. From this, we speculate that ND7 and cox3 mRNAs are extensively edited in *B. saltans*, as we found two ND7 and three cox3 gRNAs in our limited collection. Because *B. saltans* diverged from the main kinetoplastid lineage before the trypanosomatids (Blom et al., 1998a; see Fig. 7), this would provide further evidence for an ancient origin of the editing of these RNAs. In spite of this early divergence, however, gRNAs in *B. saltans* do not seem to possess 5′ terminal U extensions, as found for *T. borreli* gRNAs (Yasuhira & Simpson, 1996).

In an attempt to also identify the seven remaining orphan gRNAs and to confirm the assignment of the seven gRNAs of Figure 3, we employed an RT-PCR approach with degenerate oligonucleotides derived from the inferred edited *B. saltans* mRNA sequences and oligo (dT) as primers. However, we were unable to amplify any of the corresponding mRNA regions, in spite of numerous experiments in which a large number of different primer combinations were used. Apparently, such an analysis is hampered by the use of degenerate oligonucleotides, which is unavoidable because of the fact that the possibility of G:U basepairing in gRNA: mRNA duplexes results in ambiguities in the inferred mRNA sequences, and because of the overall low abundance of edited mt mRNAs in total RNA from *B. saltans* (Blom et al., 1998a). Therefore, the (definitive) assignment of these gRNAs must await further sequence analysis of edited mRNAs in *B. saltans*.

B. saltans minicircles are not organized in large networks

When intact, undegraded total DNA of *B. saltans* was analyzed by EM, no evidence could be obtained for the existence of a catenated network of mt DNA, under conditions that allow easy visualization of such networks in total DNA from *C. fasciculata* (Fig. 4A,B; see Blom et al., 1998a). The availability of cloned minicir-

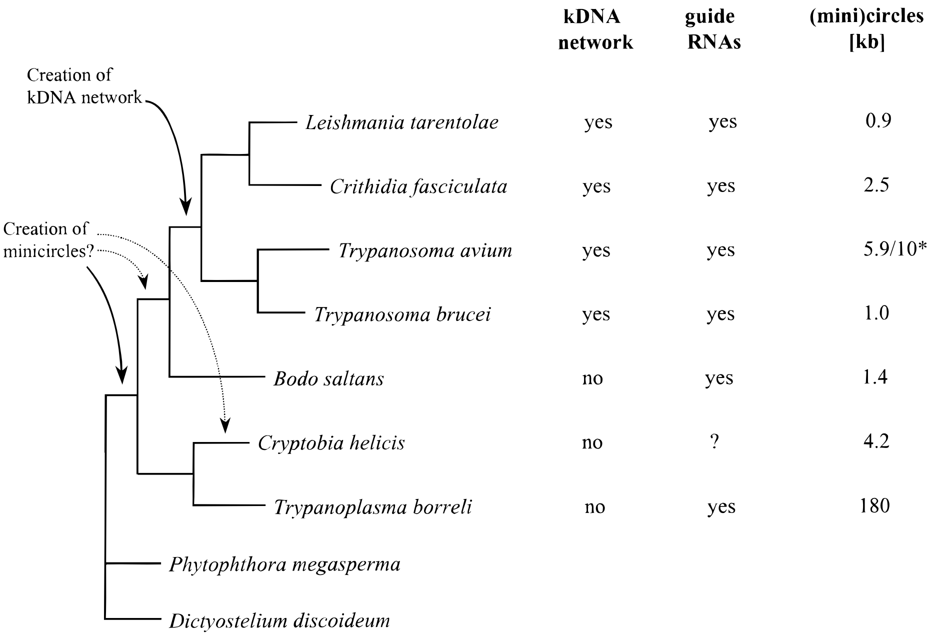


FIGURE 7. Phylogeny of kinetoplastid minicircles and minicircle networks. The phylogenetic tree was constructed as described in Materials and Methods, based on the nucleotide and amino acid sequence of a 296 nt cox2 (c)DNA fragment. The occurrence of kinetoplast DNA networks and gRNAs has been listed, the question mark indicating that the existence of gRNAs has not been formally proved for *C. helicis*. The size of the (presumed) gRNA-encoding (mini)circles has been indicated in the right column [*, depending on the strain: 10 kb in strain A1412 and 5.9 kb in strain A493 (Yurchenko et al., 1999)]. The significance of the different types of arrows is discussed in the text.

cles enabled us to further substantiate the EM analysis. We first analyzed the behavior of minicircles present in total *B. saltans* DNA during sucrose gradient sedimentation and gel electrophoresis (Figs. 5A, and 6A,B, respectively). In none of the experiments (performed with five different DNA isolates) did we find minicircles at the positions expected for a network, that is, the bottom fractions of the sucrose gradients and the slots of the agarose gels. In contrast, a substantial number of the minicircles in total DNA from *C. fasciculata* were found at these positions in control experiments (40–60%, Figs. 5B, and 6A,B). When the electrophoresis experiment was performed with *B. saltans* DNA that was 19-fold enriched for minicircles and maxicircles on CsCl-Hoechst floating density gradients, the results were essentially the same (Fig. 6C).

The existence of free 1.4-kb *B. saltans* minicircles could be easily demonstrated. First, EM analysis of total DNA revealed the presence of relaxed DNA circles of 1.4 kb in size (Fig. 4A), which were 19-fold more abundant in DNA enriched with minicircles (Fig. 4C). EM analysis of hybridizing DNA extracted from agarose gels further showed the abundant presence of two circular forms of minicircles and of a linear form (l in Fig. 6). We infer that the faster and slower moving circular forms correspond to closed and nicked circular minicircles (c and n, respectively, in Fig. 6), as closed and nicked minicircles of *C. fasciculata* migrate to the same relative positions on agarose gels (Fig. 6A,B; see Kitchin et al., 1984). Together, circular 1.4-kb minicircles made up 55–75% (depending on the DNA preparation) of the total number of minicircles present in *B. saltans* DNA, whereas 15–30% were present in the linearized form. In addition to the minicircle monomers, we also found small numbers of interlocked dimers (5–10%) and trimers (1–5%) (Figs. 4A and 6). We did not find supercoiled circles, which is a notable difference from *C. helicis*, which was shown to possess supercoiled, noncatenated minicircles (Lukes et al., 1998).

We do not know why such a relatively high percentage of the minicircles is either nicked or linearized. In view of the care that was taken to avoid shearing of the DNA, allowing, for example, the isolation of virtually intact large molecules such as the 70-kb maxicircles (Blom et al., 1998a), we infer that the extent of minicircle degradation that has occurred during isolation is limited and that the distribution of minicircle forms in our DNA preparations more or less represents the distribution in vivo. However, even if all linear minicircles present in the *B. saltans* DNA preparations were created by degradation during the isolation procedure, this could not have resulted in the complete destruction of a preexisting network. According to a mathematical model developed by Chen et al. (1995), linearization of 15–30% of the minicircles present in a kDNA network in which each minicircle is linked to three neighbors ($n = 3$) would result in the release of only 0.5–2% of the minicircles as monomeric circles, whereas similar amounts would be released as dimers and trimers. For other possible linkage numbers ($n = 4$, $n = 6$), the number of released monomeric circles predicted to arise would be virtually zero. Because our preparations contained 55–75% circular monomers, we conclude that the absence of a kDNA network in *B. saltans* (mt)DNA is not due to its degradation during DNA isolation, but rather to the fact that the minicircles are not organized in a network. The reason for the existence of minicircle dimers and trimers is unclear. However, one should realize that comparable amounts of dimers and trimers of circular mt DNAs are also found in mitochondria of mammalian cells, in which they are likely to be (side) products of DNA replication or recombination (Clayton et al., 1968).

The mt genetic system of the bodonid *B. saltans* resembles that of trypanosomatids with respect to maxicircle gene organization, editing patterns of mt mRNAs, and the existence of gRNA-containing minicircles (see Blom et al., 1998a). However, as far as the organization of the kDNA is concerned, *B. saltans* mt DNA is more similar to other bodonids such as *B. caudatus* (Hajduk et al., 1986) and the cryptobiids *C. helicis* (Lukes et al., 1998) and *T. borreli* (Lukes et al., 1994; Yasuhira & Simpson, 1996). In all these species the typical kDNA disc and the catenated kDNA network appear to be missing. In *T. borreli,* minicircles are missing altogether and the gRNAs seem to reside on a (single?) 180-kb large circular DNA molecule (Yasuhira & Simpson, 1996). In *B. caudatus* and *C. helicis*, noncatenated minicircle-like circular DNAs of 10 and 12 (Hajduk et al., 1986) and 4.2 kb (Lukes et al., 1998) have been found, but it remains to be demonstrated that these molecules indeed contain gRNA genes. This makes *B. saltans* the first organism shown to possess gRNA-containing, noncatenated minicircles (see Fig. 7). It has been proposed that the function of the kDNA network in trypanosomatid flagellates is to prevent the loss of essential minicircle-encoded gRNA genes during kDNA replication (Borst, 1991). Although this may be true for trypanosomatids, *B. saltans* seems to get by perfectly without a network and it remains to be established how the loss of essential minicircles is prevented in this organism. Possible scenarios could be minicircle redundancy, as proposed for *C. helicis*, which appears to harbor three times as many (putative) minicircles as *C. fasciculata* (Lukes et al., 1998). Alternatively, specific proteins or protein-containing structures may exist that enforce strict regulation of the minicircle replication process.

To shed light on the evolutionary history of minicircles and minicircle networks, we have incorporated the data on mt DNA organization from different trypanosomatids, bodonids, and cryptobiids into a phylogenetic tree constructed with mt cox2 gene sequences (Fig. 7; see Blom et al., 1998a), which is in good agreement with more robust trees based on nuclear-encoded rRNA gene sequences (results not shown). Only trypanosomatids possess minicircle networks, leading to the conclusion that networks arose after the divergence of the bodonid and trypanosomatid lineages. However, minicircles must have existed in earlier times, given their presence in *B. saltans* and other bodonid species. It has been hypothesized that the gRNA-encoding 180-kb molecules of *T. borreli* represent evolutionary precursors of minicircles (Yasuhira & Simpson, 1996; Simpson, 1997). Indeed, the creation of small circular DNA molecules from a large, master circle is a well known phenomenon in mitochondria of higher plants (Fauron et al., 1995). However, provided that the topology of the tree of Figure 7 is correct, such a scheme would have to lead to the assumption that minicircles were created twice during kinetoplastid evolution: once in the main lineage after branching off from the cryptobiid lineage and once in the *C. helicis* lineage after the separation from *T. borreli* (see the dashed arrows in Fig. 7). A more parsimonious scenario would therefore be that (mini)-circles have a more ancient origin in the kinetoplastid lineage (solid arrow in Fig. 7) and that the 180-kb molecules in *T. borreli* are a derived trait. If one assumes that the 10- and 12-kb DNA molecules of *B. caudatus* indeed represent minicircles, the latter scenario is supported by the position of *B. caudatus* in phylogenetic trees of Euglenozoa based on rRNA gene sequences, in which it branches off before *T. borreli* (Maslov et al., 1999). Further data on the mt DNA organization of bodonids are required to settle this issue.

MATERIALS AND METHODS

Cell culture and nucleic acid isolation

B. saltans (strain K1) was cultured as described (Blom et al., 1998a), to a cell density of $\sim 10^7$ cells/mL. Cells from 4-L cultures were spun down, resuspended in 200 mL of fresh medium, and left overnight at 4 °C in a column to allow sedimentation of the feeder bacteria. Remaining bacteria were removed by differential centrifugation. Cells were lysed and total DNA was isolated as described (Jirku et al., 1995). During the isolation procedure, shearing of the DNA was avoided as much as possible. To purify kDNA, total DNA was loaded onto a Hoechst 33258 dye-CsCl equilibrium density gradient and centrifuged in a Beckman Ti50 rotor at 45,000 for 48 h, as described (Simpson, 1979). Total *B. saltans* RNA was isolated with a Rapid RNA purification kit (Amresco), according to the manufacturer's instructions. A fraction enriched for gRNA and tRNA was prepared following tRNA isolation procedures (Greer, 1994). *C. fasciculata* [Steinert strain, (Yasuhira & Simpson, 1995)] was grown as described (Kleisen et al., 1975). *C. fasciculata* total DNA was isolated using the hot-phenol extraction method described by Borst and Fase-Fowler (1979) and mt DNA was isolated as described (Kleisen et al., 1975).

Sucrose gradients

Sucrose-gradient sedimentation was performed essentially as described (Englund, 1979; Lukes et al., 1998) with a Beckman SW28 rotor. Fractions of about 1.1 mL were collected from the bottom of the gradient. DNA was isolated from the collected fractions, spotted onto Hybond-N membranes (Amersham), and hybridized with a minicircle probe. Radioactive signals were quantified with a Storm 860 phosphorimager, and count retention was established by comparison of signals from every fraction with signals obtained from serially diluted total DNA (hybridized with the same probe).

Electron microscopy

DNA from *B. saltans* and *C. fasciculata* was spread for electron microscopy as described (Lukes et al., 1994), using bacteriophage PM2 DNA (Boehringer) as an internal size marker. Spread DNA was stained in 10^{-5} M uranyl acetate for 10 s, rinsed in 90% ethanol, and air-dried. After rotary shadowing with platinum/palladium at an angle of 7°, grids were viewed in a Philips EM 400. A grating replica was used to determine the magnification.

Electrophoresis, blotting, hybridization, and PCR

Standard agarose gel electrophoresis of DNA or RNA, Southern and Northern blotting, and hybridization procedures were performed essentially as described (Chu et al., 1986; Sambrook et al., 1989; Van der Spek et al., 1990, 1991). Prior to blotting of a DNA gel, slots were filled with low-melting-point agarose to prevent loss of DNA still present in the slots and the gel was incubated in 0.25 M HCl for 10 min to facilitate the blotting of large molecules. For PCR amplification of DNA fragments, the following protocol was used: 1 ng of recombinant DNA or 100–250 ng of total *B. saltans* DNA was incubated with 40 pmol oligonucleotides for 5 min at 95 °C, after which it was amplified in 30 cycles of 1 min at 95 °C, 1.5 min at 50 °C and 1.5 min at 74 °C. The exact annealing temperature depended on the length and GC-content of the oligonucleotides used in a particular experiment: 4 °C per G or C + 2 °C per A or T, − 5 °C. For these reactions 1 U of Taq polymerase and buffers were used according to the manufacturer's instructions (Promega). For amplification of RNA segments (RT-PCR), the PCR protocol was preceded by cDNA synthesis: 0.1 μg of *B. saltans* mRNA was denatured for 2 min at 70 °C, then immediately put on ice and added to the RT-mix containing 300 ng of the downstream primer, 10 U of reverse transcriptase (Promega), 10 U of RNAsin (Promega) and RT buffer in a total volume of 30 μL. The mixture was incubated for 1 h at 42 °C, followed by 5 min at 95 °C; 5 μL of the mixture was used for PCR essentially as described above. For *Escherichia coli* colony PCR, clones were picked and dissolved in 50 μL of H_2O and boiled for 5 min; 1 μL of this solution was then used in a PCR reaction as described above.

Oligonucleotides

Sequencing of cloned minicircle fragments

BM1: TTGTAGGGGGATCATCTTTA
BM2: ATATGAAGTGGGGGCAGCAA
BM3: TATTTAGCGCTGTTTTTGAT
BM4: AGCGGGCAGGCTATCGAACA
BM7: ATCTTTCTAATTCGGATTG
BM8: CGATTGAGCGTAGAGCATAG.

Construction of a gRNA-cDNA library

CDS1:
AAGCAGTGGTAACAACGCAGAGTACA$_{(20)}$(G/T/C)N
SMART II:
AAGCAGTGGTAACAACGCAGAGTACGCGGG
PCR primer:
AAGCAGTGGTAACAACGCAGAGT.

Assignment of gRNA gene cassette on minicircles

T7 primer: TAATACGACTCACTATAGGG
M13 reverse: AACAGCTATGACCATG
BM5: CCTGACGACACAGTACTAC
BM6: GGGTCTAGGGCACCACAG
BM10: TACTCTGCGTTGTTACCAGTGCT
BM11: TGCTCATTACAACAAATGCTGA
BM12: CGCAAACAAACATCCAATGAG
BM13: AATTACAAAACAAACCATTAGCA
BM14: AATAAACAGACCGCAACAAGAA
BM15: ATTCATTTCTAACTTATTTATTAATG
BM16: ATTATGCCACAAACAACAAGTG
BM17: CGGTGCAAAACATCATTATCTA
BM18: AACAACAACAGCATTGAAGCTA
BM19: AGGGAAAGGGGAATAAAGAAC
BM20: ACCAACACAAAACCAAAGAAGA
BM21: ACCTATAACACAACATCCAAGAT
BM22: CCAAGCATTGAGCAGTTAAAG
BM23: ACACAAGCAGTTGTACTAACAT
BM25: AACACAATCCGAATTAGAAAGAT
BM26: AACCAAAACACAAAACACACAAT
BM27: CGAACAACTAACAAACAGTAAG
BM28: TCAAACAACAAATCAATTGATATG.

Primer extension analysis

BM50: CTTGATTTTGCGTCTTCATATG
BM51: GTTCTTTTATCTTTCTAATTCGG.

Cloning and sequencing of minicircles

Total DNA from *B. saltans* was digested with either *Eco*RI or *Hin*dIII followed by electrophoresis on a 0.8% agarose gel. DNA bands migrating at 1.4 kb (representing linearized minicircles) were isolated using a DNA extraction kit (Qiagen) and cloned into the *Eco*RI or *Hin*dIII site of the pUC19 cloning vector. Clones containing 1.4-kb inserts were selected and the inserts of four of them were sequenced in their entirety using numerous oligonucleotides (sequences shown above) and ABI automatic sequencing protocols. Two of these clones (pBME40 and pBME59) contained an *Eco*RI-digested minicircle, the other two (pBMH06 and pBMH07) a *Hin*dIII-digested minicircle. The sequences of the minicircles have been deposited in GenBank under accession numbers AF 177240–177243.

Cloning, sequencing, and 5′-end determination of gRNAs

Total RNA from *B. saltans* was used as starting material for the construction of a gRNA-cDNA library with the SMART-PCR cDNA Synthesis Kit (Clontech). This procedure made use of primer CDS1, designed to anneal to the 3′ U-tail of gRNAs, to synthesize first-strand cDNA. The 3′ end of this cDNA contained the complement of the SMART II oligonucleotide as a result of template switching of the Reverse Transcriptase enzyme used in this procedure. First-strand cDNA was amplified using the PCR primer. DNA bands of 100–200 nt, the appearance of which was dependent on the addition of Reverse Transcriptase during first-strand synthesis, were isolated using an extraction kit (Qiagen) and were cloned into the pGEM-T vector (Promega). Clones were analyzed by colony-PCR (see above), making use of the CDS1 primer in combination with a vector-specific primer (either the T7 primer or the M13 reverse primer). This strategy enabled us to determine the exact size of the inserts and to eliminate primer-dimer artifacts. Inserts were sequenced following ABI automatic sequencing protocols. Sequences of (putative) gRNAs are given in Figure 2 and have been deposited in GenBank under accession numbers AF 177244–177257. The 5′ end points of the two ND5 gRNAs encoded by minicircles pBMH06 and pBMH07 were determined by primer extension with 8 U of AMV RT, 25 ng of small *B. saltans* RNA [prepared following tRNA isolation procedures according to Greer (1994)] and 1 ng of oligonucleotides BM50 or BM51, essentially as described (Van der Spek et al., 1990).

Cloning of gRNA gene-containing minicircle fragments

Minicircle fragments were amplified by PCR using oligonucleotides derived from gRNA sequences (BM10 through BM28), in combination with either BM5 or BM6, two oligonucleotides with opposite orientations, derived from a conserved sequence domain of the *B. saltans* minicircle (see Fig. 1A). Amplified fragments of ~450 nt were run on a 0.8% agarose gel, isolated, and cloned into the pGEM-T vector (Promega). The inserts of positive clones were sequenced by ABI automated sequencing. The obtained sequences were used for the assignment of the gRNA gene in question to either cassette I or II (see Fig. 1C) and for verification of (part of) the gRNA sequence. The sequences have been deposited in GenBank under accession numbers AF 177258–177271.

Construction of phylogenetic trees

For the construction of phylogenetic trees, we analyzed cox2 cDNA sequences of *L. tarentolae* (De la Cruz et al., 1984), *C. fasciculata* (Van der Spek et al., 1989), *T. brucei* (Hensgens et al., 1984; Benne et al., 1986), *T. borreli* (Lukes et al., 1994), *B. saltans* (Blom et al., 1998a), *T. avium* (Blom et al., 1998a), and *C. helicis* (Blom et al., 1998a), using the Clustal algorithm in the program MEGAlign (Version 3.10a, DNAStar Inc., USA). The maximum parsimony tree was constructed on the basis of combined matrices of (c)DNA and amino acid data.

ACKNOWLEDGMENTS

We thank David Dolezel and Jana Fisáková for skillful experimental assistance, Paul Englund for providing the probe pDP312, Jan van Marle for assistance with the EM analysis, Dave Speijer for stimulating discussions and critical reading of the manuscript, and Wendy van Noppen for editorial comments. The research of the Amsterdam group was supported by the Netherlands Foundation for Chemical Research (SON), which was subsidized by the Netherlands Foundation for Scientific Research (NWO). The Czech group was supported by a grant from the Czech Academy of Sciences No. A6022903.

Received September 14, 1999; returned for revision October 14, 1999; revised manuscript received October 29, 1999

REFERENCES

Alfonzo JD, Thiemann O, Simpson L. 1997. The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. *Nucleic Acids Res 25*:3751–3759.

Arts GJ, Benne R. 1996. Mechanism and evolution of RNA editing in kinetoplastida. *Biochim Biophys Acta 1307*:39–54.

Avila HA, Simpson L. 1995. Organization and complexity of minicircle-encoded guide RNAs from *Trypanosoma cruzi*. *RNA 1*:939–947.

Benne R, van den Burg J, Brakenhoff JPJ, Sloof P, van Boom JH, Tromp MC. 1986. Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell 46*:819–826.

Birkenmeyer L, Sugisaki H, Ray DS. 1987. Structural characterization of site-specific discontinuities associated with replication origins of minicircle DNA from *Crithidia fasciculata*. *J Biol Chem 262*:2384–2392.

Blom D, de Haan A, van den Berg M, Sloof P, Jirku M, Lukes J, Benne R. 1998a. RNA editing in the free-living bodonid *Bodo saltans*. *Nucleic Acids Res 26*:1205–1213.

Blom D, de Haan A, van den Berg M, Sloof P, Jirku M, Lukes J, Benne R. 1998b. *Nucleic Acids Res 26*:5539.

Borst P. 1991. Why kinetoplast DNA networks? *Trends Genet 7*:139–141.

Borst P, Fase-Fowler F. 1979. The maxicircle of *Trypanosoma brucei* kinetoplast DNA. *Biochim Biophys Acta 565*:1–12.

Brooker BE. 1971. Fine structure of *Bodo saltans* and *Bodo caudatus* (Zoomastigophora; Protozoa) and their affinities with Trypanosomatidae *Bull Brit Museum [Nat Hist] 22*:89–102.

Brugerolle G, Lom J, Nohyncová E, Joyon L. 1979. Comparaison et évolution des structures cellulaires chez plusieurs espèces de Bodonidés et Cryptobiidés appartenant aux genres *Bodo*, *Cryptobia* et *Trypanoplasma* (Kinetoplastida, Mastigophora). *Protistologica 15*:197–221.

Chen J, Rauch CA, White JH, Englund PT, Cozzarelli N. 1995. The topology of the kinetoplast DNA network. *Cell 80*:61–69.

Chu G, Vollrath D, Davis RW. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science 234*:1582–1585.

Clayton DA, Smith CA, Jordan JM, Teplitz M, Vinograd J. 1968. Occurrence of complex mitochondrial DNA in normal tissues. *Nature 220*:976–979.

De la Cruz VF, Neckelmann N, Simpson L. 1984. Sequences of six genes and several open reading frames in the kinetoplast maxicircle DNA of Leishmania tarentolae. *J Biol Chem 259*:15136–15147.

Englund PT. 1979. Free minicircles of kinetoplast DNA in *Crithidia fasciculata*. *J Biol Chem 254*:4895–4900.

Fauron C, Casper M, Gao Y, Moore B. 1995. The maize mitochondrial genome: Dynamic, yet functional. *Trends Genet 11*:228–235.

Fernandes AP, Nelson K, Beverley SM. 1993. Evolution of nuclear ribosomal RNA in kinetoplastid protozoa: Perspectives on the age and origins of parasitism. *Proc Natl Acad Sci USA 90*:11608–11612.

Greer GL. 1994. Processing of transfer RNA precursors. In: Higgins SJ, Hames BD, eds. *RNA processing, a practical approach*. Vol. II. Oxford: Oxford University Press. pp 173–209.

Hajduk SL, Sabatini RS. 1998. Mitochondrial mRNA editing in kinetoplastid protozoa. In: Grosjean H, Benne R, eds. *Modification and editing of RNA*. Washington DC: ASM Press. pp 377–393.

Hajduk SL, Siqueira AM, Vickerman K. 1986. Kinetoplast DNA of *Bodo caudatus*: A noncatenated structure. *Mol Cell Biol 6*:4372–4378.

Hensgens LAM, Brakenhoff J, de Vries BF, Sloof P, Tromp MC, van Boom JH, Benne R. 1984. The sequence of the gene for cytochrome *c* oxidase subunit I, a frameshift containing gene for cytochrome *c* oxidase subunit II and seven unassigned reading frames in *Trypanosoma brucei* mitochondrial maxicircle DNA. *Nucleic Acids Res 12*:7327–7344.

Hinz S, Göringer HU. 1999. The guide RNA database (3.0). *Nucleic Acids Res 27*:168.

Jirku M, Kolesnikov AA, Benada O, Lukes J. 1995. Marine fish and ray trypanosomes have large kinetoplast minicircles DNA. *Mol Biochem Parasitol 73*:279–283.

Kitchin PA, Klein VA, Fein BI, Englund P. 1984. Gapped minicircles. A novel replication intermediate of kinetoplast DNA. *J Biol Chem 259*:15532–15539.

Kleisen CM, Borst P, Weijers PJ. 1975. Properties of the intact multicircular complex from *Crithidia fasciculata*. *Biochim Biophys Acta 390*:155–167.

Lukes J, Arts GJ, Van den Burg J, de Haan A, Opperdoes F, Sloof P, Benne R. 1994. Novel pattern of editing regions in mitochondrial transcripts of the cryptobiid *Trypanoplasma borreli*. *EMBO J 13*:5086–5098.

Lukes J, Jirku M, Avliyakulov N, Benada O. 1998. Pankinetoplast DNA structure in a primitive bodonid flagellate, *Cryptobia helicis*. *EMBO J 17*:838–846.

Lukes J, Jirku M, Dolezel D, Král'ová L, Hollar L, Maslov DA. 1997. Analysis of ribosomal RNA genes suggests that trypanosomes are monophyletic. *J Mol Evol 44*:521–527.

Maslov DA, Avila HA, Lake JA, Simpson L. 1994. Evolution of RNA editing in kinetoplastid protozoa. *Nature 368*:345–348.

Maslov DA, Hollar L, Haghighat P, Nawathean P. 1998. Demonstration of mRNA editing and localization of guide RNA genes in kinetoplast-mitochondria of the plant trypanosomatid *Phytomonas serpens*. *Mol Biochem Parasitol 93*:225–236.

Maslov DA, Simpson L. 1994. RNA editing and genomic organization in the cryptobiid kinetoplastid protozoan, *Trypanoplasma borreli*. *Mol Cell Biol 14*: 8174–8182.

Maslov DA, Yasuhira Y, Simpson L. 1999. Phylogenetic affinities of *Diplonema* within the Euglenozoa as inferred from the SSU rRNA gene and partial COI sequences. *Protist 150*:33–42.

Ntambi J, Marini J, Bangs J, Hajduk S, Jimenez H, Kitchin P, Klein V, Ryan K, Englund P. 1984. Presence of a bent helix in fragments of kinetoplast DNA minicircles from several trypanosomatid species. *Mol Biochem Parasitol 12*:273–287.

Pollard VW, Rohrer SP, Michelotti EF, Hancock K, Hajduk SL. 1990. Organization of minicircle genes for guide RNAs in *Trypanosoma brucei*. *Cell 63*:783–790.

Ray D. 1989. Conserved sequence blocks in kinetoplast DNA minicircles from diverse species of trypanosomes. *Mol Cell Biol 9*:1365–1367.

Ray DS, Hines JC, Sugisaki H, Sheline C. 1986. kDNA minicircles of the major sequence class of *Crithidia fasciculata* contain a single region of bent helix widely separated from the two origins of replication. *Nucleic Acids Res 14*:7953–7965.

Riley GR, Corell RA, Stuart K. 1994. Multiple guide RNAs for identical editing of *Trypanosoma brucei* apocytochrome *b* mRNA have an unusual minicircle location and are developmentally regulated. *J Biol Chem 269*:6101–6108.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Shapiro TA, Englund PT. 1995. The structure and replication of kinetoplast DNA. *Annu Rev Microbiol 49*:117–143.

Simpson L. 1979. Isolation of maxicircle component of kinetoplast DNA from hemoflagellate protozoa. *Proc Natl Acad Sci USA 76*:1585–1588.

Simpson L. 1987. The mitochondrial genome of kinetoplastid protozoa: Genomic organization, transcription, replication and evolution. *Annu Rev Microbiol 41*:363–382.

Simpson L. 1997. The genomic organization of guide RNA genes in kinetoplastid protozoa: Several conundrums and their solutions. *Mol Biochem Parasitol 86*:133–141.

Stuart K, Allen TE, Heidmann S, Seiwert SD. 1997. RNA editing in kinetoplastid protozoa. *Microbiol Mol Biol Rev 61***:**105–120.

Sturm NR, Simpson L. 1991. *Leishmania tarentolae* minicircles of different sequence classes encode single guide RNAs located in the variable region approximately 150 bp from the conserved region. *Nucleic Acids Res 22*:6277–6281.

Thiemann OH, Maslov DA, Simpson L. 1994. Disruption of RNA editing in *Leishmania tarentolae* by the loss of minicircle-encoded guide RNA genes. *EMBO J 13*:5689–5700.

Van der Spek H, Arts GJ, Van den Burg J, Sloof P, Benne R 1989. The nucleotide sequence of mitochondrial maxicircle genes of *Crithidia fasciculata*. *Nucleic Acids Res 17*:4876.

Van der Spek H, Arts GJ, Zwaal RR, Van den Burg J, Sloof P, Benne R. 1991. Conserved genes encode guide RNAs in mitochondria of *Crithidia fasciculata*. *EMBO J 10*:1217–1224.

Van der Spek H, Speijer D, Arts GJ, Van den Burg J, Van Steeg H, Sloof P, Benne R. 1990. RNA editing in transcripts of the mitochondrial genes of the insect trypanosome *Crithidia fasciculata*. *EMBO J 9*:257–262.

Vickerman K. 1977. DNA throughout the single mitochondrion of a kinetoplastid flagellate: Observations on the ultrastructure of *Cryptobia vaginalis*. *J Protozool 24*:221–233.

Wiemer EAC, Hannaert V, Van den IJsel PRLA, Van Roy J, Opperdoes FR, Michels PAM. 1995. Molecular analysis of glyceraldehyde-3-phosphate dehydrogenase in *Trypanoplasma borreli*: An evolutionary scenario of subcellular compartmentation in Kinetoplastida. *J Mol Evol 40*:443–454.

Yasuhira S, Simpson L. 1995. Minicircle-encoded guide RNAs from *Crithidia fasciculata*. *RNA 1*:634–643.

Yasuhira S, Simpson L. 1996. Guide RNAs and guide RNA genes in the cryptobiid kinetoplastid protozoan, *Trypanoplasma borreli*. *RNA 2*:1153—1160.

Yurchenko V, Hobza R, Benada O, Lukes J. 1999. *Trypanosoma avium*: Large minicircles in the kinetoplast DNA. *Exp Parasitol 92*:215—218.