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Guide RNAs and guide RNA genes in the cryptobiid kinetoplastid protozoan, *Trypanoplasma borreli*

SHINJI YASUHIRA[1,4] and LARRY SIMPSON[1,2,3]

[1] Howard Hughes Medical Institute, University of California, Los Angeles, California 90095-1662, USA
[2] Department of Molecular, Cellular and Developmental Biology, University of California, Los Angeles, California 90095-1662, USA
[3] Department of Medical Microbiology and Immunology, University of California, Los Angeles, California 90095-1662, USA

ABSTRACT

***Trypanoplasma borreli* belongs to the bodonid/cryptobiid group of kinetoplastid protozoa, which represents a sister group to the trypanosomatids. RNA transcripts from several mitochondrial genes in this organism undergo the trypanosomatid type of uridine addition/deletion RNA editing. A guide RNA (gRNA) cDNA library was constructed and five gRNAs were identified, one for editing the ribosomal protein S12 mRNA, three for editing the cytochrome oxidase subunit I mRNA, and one for editing the cytochrome *b* mRNA. All of the gRNAs contained nonencoded oligo[U] sequences at the 3′ end, as is common with gRNAs in trypanosomatids, but also contained nonencoded oligo[U] sequences at the 5′ end. The mechanism for addition of the 5′ nonencoded oligo[U] sequence and the function of this sequence are unknown. The *T. borreli* gRNAs were shorter (25–35 nt, excluding the 5′ oligo[U]) than gRNAs in trypanosomatids (45–50 nt), indicating a smaller size of editing blocks in this organism. Genomic sequences for two gRNAs were cloned and sequenced. These two gRNA-encoding sequences were shown to originate from the 180-kb Component I molecules, which represent a possible homologue of minicircle DNA in trypanosomatids, and not from the 80-kb Component II molecules, which contain the structural genes and cryptogenes.**

Keywords: cryptobiid; GenBank accession numbers: gg009 (U47932), gg043 (U47933); guide RNAs; kinetoplastid DNA; RNA editing

INTRODUCTION

Trypanoplasma borreli is a fish parasite belonging to the bodonid/cryptobiid group of kinetoplastid protozoa, which represents a sister group to the trypanosomatids. Organisms in this group do not have a network composed of catenated minicircle DNA molecules in their mitochondria, as is common in trypanosomatids (Hajduk et al., 1986; Maslov & Simpson, 1994). The mitochondrial genome of *T. borreli* consists of at least two classes of DNA molecules, which have been termed Component I and Component II (Maslov & Simpson, 1994). Component I DNA (170–200 kb) contains 1-kb repeat units and was proposed to represent a homologue of minicircle DNA in trypanosomatids, whereas Component II DNA (80–90 kb) encodes rRNAs and structural genes and represents a possible maxicircle homologue. The gene order in Component II DNA in *T. borreli* is completely different from that in trypanosomatids, consistent with the relatively distant evolutionary relationship. It was also shown that transcripts of several structural genes in this organism are edited extensively by uridine insertion- and deletion-type RNA editing (Lukes et al., 1994; Maslov & Simpson, 1994). To understand the evolution of the U-addition/deletion-type RNA editing in the kinetoplastid protozoa, it would be very informative to analyze in detail the nature and genomic organization of guide RNAs (gRNAs) in this species.

In the present study, a gRNA-cDNA library was constructed and several specific gRNAs that guide the editing of edited transcripts were identified. The genomic organization of the genes encoding these gRNAs was investigated. The results have significant implications for the evolution of RNA editing in the kinetoplastid protozoa.

Reprint requests to: Larry Simpson, Howard Hughes Medical Institute—UCLA, 6780 MacDonald Research Laboratories, 675 Circle Dr. S., Los Angeles, California 90095-1662, USA; e-mail: simpson@sunls.hhmi.ucla.edu.

[4] Present address: Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-77, Japan.

RESULTS

Construction of a gRNA-cDNA library from *T. borreli*

It was shown previously that short heterogeneously sized RNA molecules that can be 5′-capped with guanyltransferase and [α-^{32}P]GTP exist in steady-state total cell RNA from *T. borreli* (Maslov & Simpson, 1994). In trypanosomatids, the mitochondrial gRNAs represent the major small RNA species that can be 5′-capped with guanyltransferase due to the presence of di- or tri-phosphates at the 5′ ends of these transcripts (Blum et al., 1990; Blum & Simpson, 1990). The GTP-labeled *T. borreli* RNAs are slightly shorter than the gRNAs from *Leishmania tarentolae* or *Crithidia fasciculata*.

A C-tail was added at the 3′ end of the gRNAs with poly[A] polymerase, and cDNA synthesis was performed using oligo[dG] as a primer. After anchor ligation, the cDNA was PCR-amplified with the combination of a $[dG]_{10}[dA]_5$ oligonucleotide and an antisense-anchor oligonucleotide. This method differed somewhat from that used previously to obtain gRNA libraries from *L. tarentolae* (Maslov & Simpson, 1992; Thiemann et al., 1994) and *C. fasciculata* (Yasuhira & Simpson, 1995), due to the shorter 3′ oligo[U] tails in the *T. borreli* gRNAs.

Characterization of random clones from the gRNA library

Because isolation of a kinetoplast-mitochondrial fraction from *T. borreli* proved difficult, total cellular RNA was used for the construction of the gRNA library. For this reason, the gRNA library was expected to contain a certain number of cytoplasmic RNA-derived non-gRNA clones.

The 86 random clones analyzed from the gRNA library could be divided into four groups according to the results of a BLAST database homology search (data not shown). Group A clones (11 clones) showed a high similarity with the sense strand sequences of known rRNAs, mRNAs, or tRNAs from *T. borreli* or related organisms. Group B clones (5 clones) showed a high similarity with the central region of the *Sca* I repeat unit sequence from Component I DNA of *T. borreli*. Group C clones (16 clones) showed modest similarities with the antisense strand sequences of known edited mRNAs from *T. borreli*. Clones in Group D (54 clones) did not show significant similarity with any known sequences.

The Group C clones represented good candidates for *T. borreli* gRNAs. Interestingly, all 16 clones in Group C had oligo[U] sequences at the 5′ ends as well as the 3′ ends, whereas none of the clones in Group A had 5′ oligo[U] sequences (see below). Because only a few edited mitochondrial mRNA sequences are known from *T. borreli*, it is possible that some Group D clones could also represent gRNAs for unidentified editing domains. In this regard, 32 of 54 Group D clones had 5′ oligo[U] sequences, which we show below are characteristic of gRNAs from this organism.

A gRNA-cDNA library was also constructed from total RNA of *C. fasciculata* by the same procedure. No clones with 5′ oligo[U] sequences were observed in this library (data not shown), indicating that the appearance of 5′ oligo[U] sequences in the *T. borreli* gRNAs is not an artifact of the cloning procedure.

Identification of five gRNAs

Each clone from the gRNA library was compared with the known edited sequences (RPS12, COI, Cyb) from *T. borreli* using the modified BestFit local alignment program from the GCG package (Devereux et al., 1984). Five clones (g009, g034, g040, g043, and g085) with the highest BestFit quality scores were selected for further analysis. All of these clones belonged to Group C and had 5′ oligo[U] sequences. Northern blot analysis was used to establish the presence of these RNAs in total cell steady-state RNA (data not shown). Alignments of the five clones with the cognate edited mRNA sequences are shown in Figure 1. In the absence of knowledge about the localization and length of the anchor sequences, the significance of the mismatches at the 5′ ends of several gRNAs (g043, g034, g040) is uncertain. It is clear that the 5′ oligo[U] sequences do not extend the duplexes. The single nucleotide mismatch in the g043 coding sequence could represent a cloning or PCR artifact.

The five identified gRNAs contain nonencoded 5′ oligo[U] sequences

The existence of oligo[U] sequences at the 5′ termini of gRNAs from *T. borreli* is novel. A 5′ RACE cloning procedure was used to further analyze the 5′-terminal sequences of these gRNAs. Several clones were selected for each gRNA and sequenced. As shown in Figure 2, all clones analyzed had 5′ oligo[U] sequences of heterogeneous lengths. The precise locations of the junction sites between the oligo[U] sequences and the guiding sequence also showed some variability.

Two *Msp* I fragments from total cell DNA hybridizing to two gRNA clones (g009 and g043) (Fig. 3) were obtained by gel elution and were cloned and sequenced. The alignments of these sequences with the gRNA sequences shown in Figure 4 indicate that the 5′ oligo[U] gRNA sequences are not encoded genomically. The alignments also showed the existence in some clones of several additional nonencoded nucleotides situated between the oligo[U] cap and the encoded sequence. These nonencoded nucleotides may

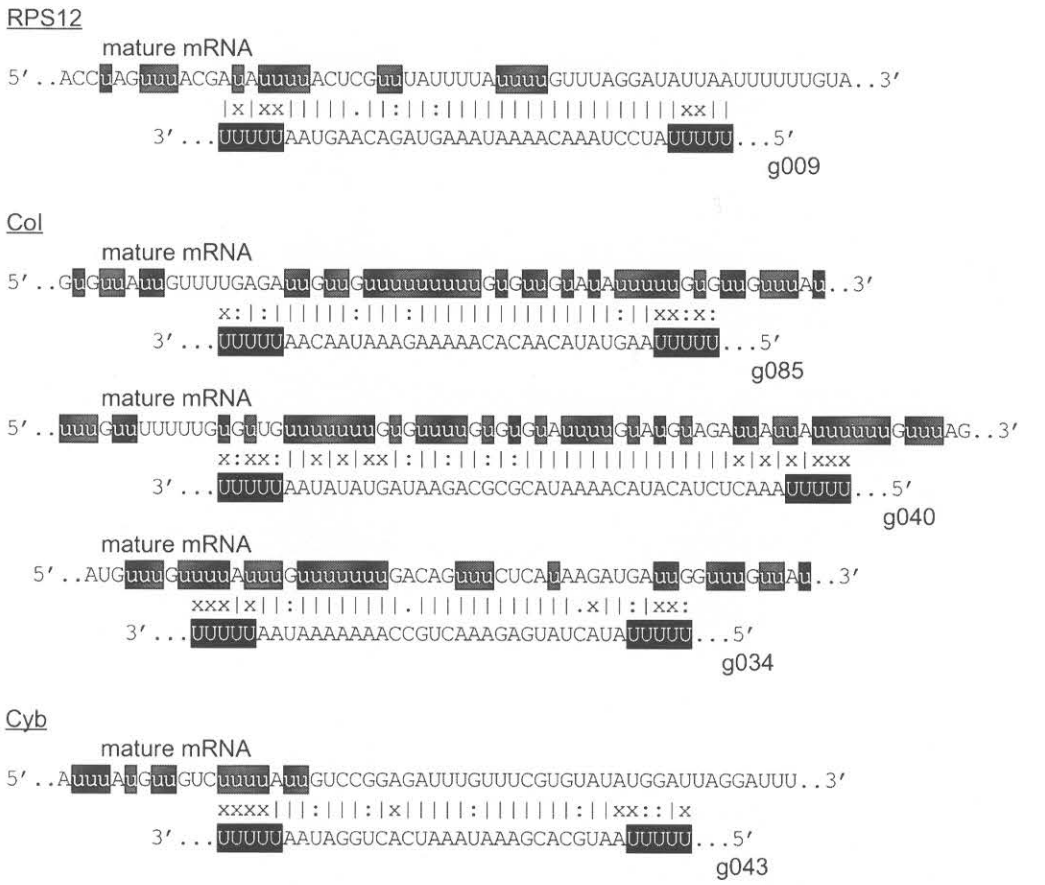

FIGURE 1. Identification of five gRNAs mediating the editing of ribosomal protein S12 (RPS12) cytochrome oxidase subunit I (COI) and cytochrome *b* (Cyb) mRNAs. The newly identified five gRNAs are shown together with their cognate mRNAs. Uridines added by editing are shown as a white "u" on black. Canonical base pairs are indicated by a line (|), G-U pairs by a colon (:), and C-A pairs by a period (.). "X" indicates a mismatch. Note the presence of a 3′ nonencoded oligo[U] tail (dark inversion), as is found in trypanosomatid gRNAs, and also the presence of a 5′ oligo[U] sequence (dark inversion), which is unique.

represent polymerase errors or may indicate the existence of a polymorphic gRNA gene family. Analysis of additional gRNA genes is required to distinguish between these possibilities.

The identified gRNA genes are not encoded by the *Sca* I repeats of the mitochondrial Component I DNA

Maslov and Simpson (1994) suggested previously that the 1-kb *Sca* I repeat units from Component I DNA may encode gRNAs, based on their finding that GTP-capped RNA hybridized to this sequence. Southern hybridizations were performed using oligonucleotide probes for the five identified gRNAs. None of the gRNA-specific probes hybridized to the 1-kb *Sca* I repeat fragments (data not shown). The absence of similarity of the flanking sequences with *Sca* I repeat sequences was shown directly by sequencing the two cloned gRNA genes, as shown in Figure 3.

Several clones from the gRNA cDNA library were found to hybridize to the central region of the *Sca* I repeat (Group B clones, see above), and four of five of these clones had 5′ oligo[U] caps (data not shown). It is likely, but not yet proven, that these clones represent a gRNA encoded by the *Sca* I repeat. Confirmation would require the identification of a cognate edited mRNA sequence.

Localization of two gRNA genes in Component I mitochondrial DNA

Pulsed field gel electrophoresis was used to identify the genomic localization of the two cloned gRNA genes. Maslov and Simpson (1994) showed previously that the circular 80-kb Component II DNA contained the structural genes and represented the maxicircle homologue, whereas the approximately 200-kb circular Component I DNA contained repetitive elements, including the 1-kb *Sca* I repeat, and represented a possible minicircle homologue. In order to linearize these DNA molecules and permit separation by pulsed field gel electrophoresis, the agarose blocks containing the lysed cells were subjected to gamma irradiation of increasing

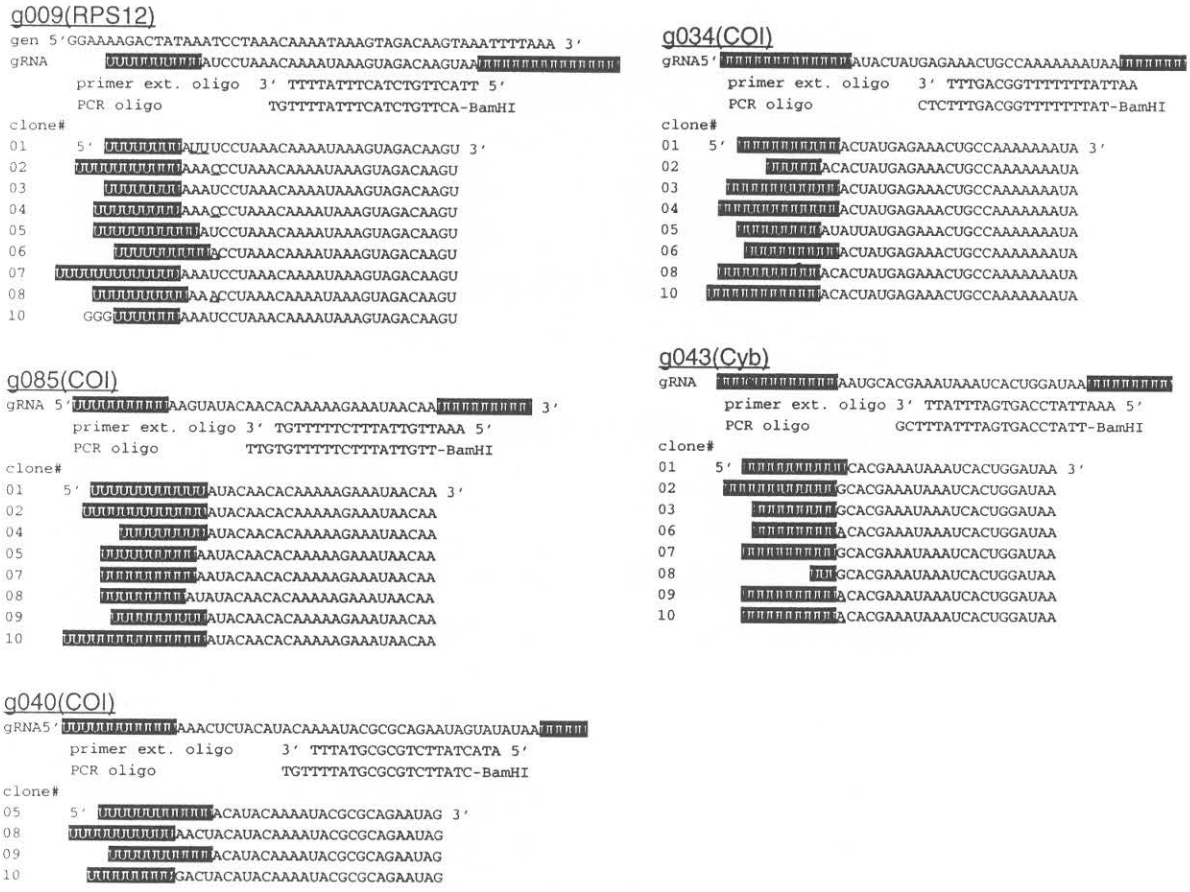

FIGURE 2. Analysis of 5′ end structure of gRNAs. Primer extension products obtained using gRNA-specific oligonucleotides were cloned by a 5′ RACE procedure, and several clones for each gRNA were sequenced. 5′ and 3′ oligo[U] sequences are highlighted, and, for g009 and g043, nucleotides that do not agree with genomic sequences are underlined. The presence of 5′ oligo[U] sequences was confirmed in all five gRNAs. Oligonucleotides used in the primer extension and PCR are shown in the Materials and methods.

doses prior to electrophoresis (Beverley, 1989). Under these pulse time conditions, nuclear chromosomes remain unresolved at the top of the gel, as shown by hybridization with a GAPDH gene fragment (Wiemer et al., 1995) (Fig. 4). Hybridization of the blot with a *Sca* I repeat from Component I DNA gave rise to a 180-kb linearized band after 200–500 Gy irradiation, whereas hybridization with a cloned Component II fragment gave rise to an 80-kb linearized band after the same amount of irradiation. Hybridization with the two cloned gRNA-encoding sequences, gg009 and gg043, yielded the same hybridization profile as the Component I probe, indicating that the gRNA genes are localized on the 180-kb Component I DNA molecules.

Relative abundance of the different mitochondrial genomic components

The relative abundance of the different mitochondrial genomic components was determined by quantitative dot blot analysis (Fig. 5). A plasmid was constructed containing, in tandem, a GAPDH nuclear gene fragment, a *Sca* I repeat unit, a COIII gene fragment from Component II DNA, and the cloned gRNA gene fragment, gg009. This was used as a reference DNA (see the Materials and methods). Table 1 summarizes the abundance of each component, assuming that there are four copies of the GAPDH gene in a single *T. borreli* cell (Wiemer et al., 1995), as in *Trypanosoma brucei*. The nuclear genome size was calculated to be 1.2×10^8 bp from the amount of DNA applied and the hybridization with the GAPDH probe. Based on these values, the *Sca* I repeat sequences were estimated to represent about 25% of total cellular DNA and the ratio of the number of gg009 gRNA-encoding sequences to that of the *Sca* I repeat units was approximately 1/1,000.

DISCUSSION

We have identified from a total cell cDNA library five putative gRNAs in *T. borreli* that could potentially mediate the editing of one block in RPS12 mRNA, three blocks in COI mRNA, and one block in Cyb mRNA.

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gg009     GTATAATGGT ACATTAATCG TGGAAAGAGT GATACCCCAT ATAACATATA
gg043                              CCA GGGGGCCTGT TAGGAAATAA

gg009     TGATATTTAT GTTAGTATAA GTTACTTTAC TCAGCAGTAA TACGTCATAT
gg043     CTACTAGTAC TCAATGGTAC ATGTATATAT CCATATATAT ACACTTTTAA

gg009     CTCTTACTCA TTCCAGTATG GGTGTTAAAC GTAATTAGAA CCATTAAGTT
gg043     ACTTATGTAA ACTTACGCGT AATATAGGGA CTCATATTAG TTTTTTAATA

gg009     ATCTTTTGTG GGGGTAATTA TTTTAAGGGG GGATTACTGA GTAGGGGGAT
gg043     CAGGAGTAAT ACATTACTTG ACCAGGTAGC ATTACCAGGA TATACTGTAT

gg009     GTGTATGTTA TATTATGCAA GATCAGTCTA TATAGGGGTC CCTTAATATC
gg043     TTTTATATTA CAGAGACCGT ACAGGTGGTA TATAAAAAAT AGAGGCTTAT

gg009     GTTGTGTATT TTCGTCATCA TAAAGATAGT TTACGTATGA TGTAAGGCAT
gg043     GGTTCTACAT TAGGAGGACA TACTTAAGTT GTCCTTAATA TAAGATATTC

gg009     ATTTTGCACA CCATATAGAT AATGTGGGTA GATACTGCTC TTGATACAAT
gg043     ATAATTTGTA TAAATGCGTT CGCTATAAGG ATCGATATGG AGGTAACTGG

gg009     GGATATATAG GGAGTAAAAC TTCTAGAATC CCAGGTAAGT GCATACTAAG
gg043     ATTGGTTACC TTAATCCCTA AGGGTAAGGA TTACGTAGGT TACTAAAATA

gg009     GACCTGGAAG CATGCTAACA TAGGCTTTAT TGGGTACAGA GGGAAAAGAC
gg043     GTCCATAGGG TAGTATATGG TAGGCTTTAT TATTTAAAAC TTTGTGGGAA

gg009     TATAAATCCT AAACAAAATA AAGTAGACAA GTAAATTTTA AACCCAATTT
gg043     TTAATGCACG AAATAAATCA CTGGATAATA AAATTCATAA TTATAATAGG
          gRNAs
gg009     CATAAGGCTA TAGAATCAAA AGTTATAAGT AATACATATT TTTATATAAA
gg043     GGGAGTTCAT ATTTGAAACT TTAAAACTAC AGACGCCTCC CGG

gg009     CAATTATGTT ATTCATTATG TATGACTCCG G
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FIGURE 3. Genomic sequences for two gRNAs. Genomic *Msp* I fragments, which hybridized to g009 and g043 gRNA-specific probes, were cloned from a partial genomic library and sequenced. The gRNA coding region is shown underlined. Note that the 5′ oligo[U] sequences of the two gRNAs are not genomically encoded. An 11-nt sequence located approximately 20 nt upstream of the gRNA coding region and conserved between the two genomic sequences is boxed; this may represent a possible regulatory sequence, but this must be confirmed by examining additional genomic sequences.

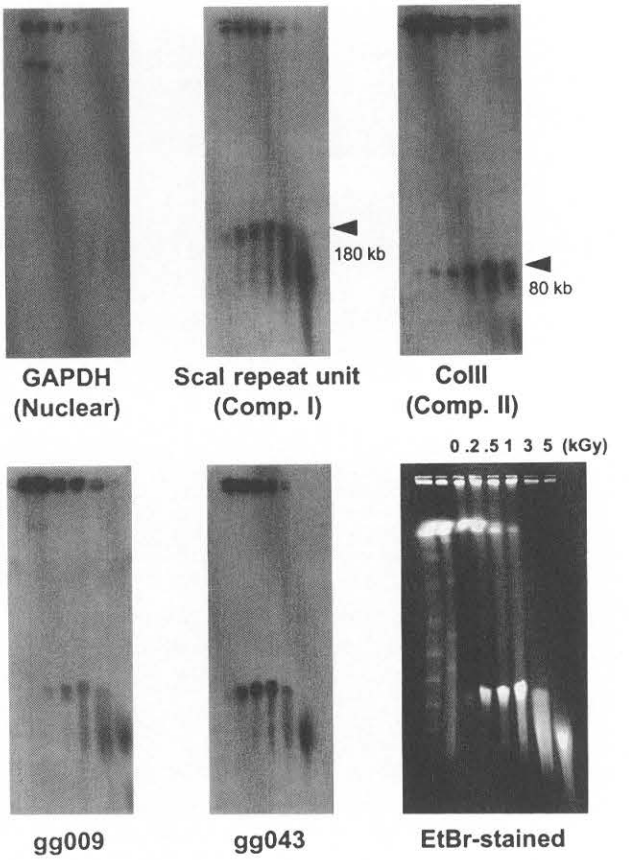

FIGURE 4. Localization of the two gRNA-encoding sequences in Component I DNA of the mitochondrial genome. DNA from *T. borreli* cells, which were embedded in agarose blocks, was irradiated with increasing doses of γ-ray emitted from a ^{60}Co source and resolved with CHEF gel electrophoresis. The blot was probed for either the GAPDH gene (cloned fragment of gene, nuclear genome), the *Sca* I 1-kb repeat (cloned 1 kb repeat fragment, Component I), the COIII gene (cloned Component II), the gg009 gRNA gene, or the gg043 gRNA gene. The last panel is the ethidium bromide-stained gel. Lane 1, lambda DNA ladder; lane 2, *S. cerevisiae* chromosomes.

These gRNAs possess the nonencoded heterogeneous 3′ oligo[U] tail characteristic of all known trypanosomatid gRNAs, which is due to the action of a mitochondrial 3′ uridylyl transferase. They also have a novel feature—a 5′ nonencoded oligo[U] cap sequence. The mechanism for the posttranscriptional addition of nucleotides to the 5′ termini of the gRNAs is not known, nor is the genetic function of such a sequence. In addition, the gRNAs can be 5′-end labeled with [α-^{32}P]GTP and guanylyl transferase, suggesting the presence of a 5′ di- or triphosphate, which may possibly indicate a primary transcript. We speculate that the 5′ oligo[U] cap could be the result of a *trans*-splicing event or an RNA ligation, but additional experimental work is required to investigate this phenomenon.

The mitochondrial DNA in *T. borreli* was shown previously (Maslov & Simpson, 1994) to consist of two classes of circular molecules: 80-kb Component II molecules, which encode rRNA genes, structural genes, and cryptogenes; and approximately 200-kb Component I molecules, which encode a 1-kb *Sca* I repeat sequence. The *Sca* I repeat was shown to hybridize to a class of small RNA transcripts that could be 5′-capped with [α-^{32}P]GTP and guanylyl transferase, which were thought to represent gRNAs (Maslov & Simpson, 1994). We have shown that the five identified gRNAs are not transcribed from the *Sca* I repeat, but nevertheless are encoded by a class of approximately 180-kb circular molecules. We have also identified several cDNA clones derived from the *Sca* I repeats. Although it is not known whether these *Sca* I transcripts represent func-

TABLE 1. Relative abundance of cellular DNA components.

Components	Estimated copy numbers
GAPDH gene (nuclear genome)	4[a]
Sca I repeat unit (Component I)	4×10^4
Co III gene (Component II)	2×10
gg009 (Component I)	5×10

[a] From (Wiemer et al., 1995)

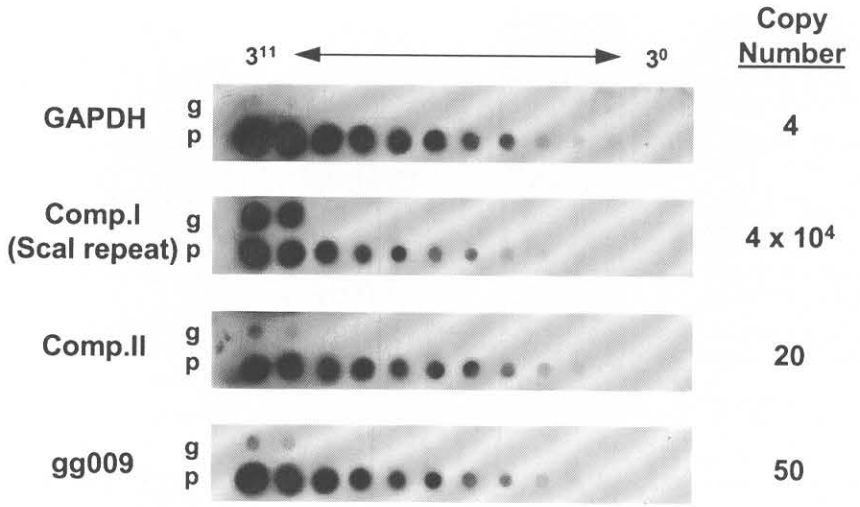

FIGURE 5. Relative abundance of each of the cellular DNA components in *T. borreli*. A serial dilution of *Sca* I-digested genomic DNA from *T. borreli* (row g) and a reference plasmid, pREF (row p), on a nylon membrane were probed with the same specific probes for each of the cellular DNA components used in Figure 4.

tional gRNAs, the similar size and the presence of a nonencoded 5′ oligo[U] cap suggest that the previously reported hybridization of small gRNA-like molecules to the *Sca* I repeats (Maslov & Simpson, 1994) was due to the existence of these transcripts.

In addition, we showed that there are approximately 1,000 times more copies of the *Sca* I repeat than the individual identified gRNA genes. Furthermore, multiple *Sca* I repeat clones were sequenced and no substantial sequence heterogeneity was observed, nor were any sequence similarities observed between the *Sca* I repeat and the two cloned Component I fragments that encoded identified gRNAs (data not shown). The 180-kb size of the Component I molecules, combined with the 1,000 to 1 ratio of *Sca* I repeat sequences to specific gRNA genes, together suggest that the Component I molecules are heterogeneous, but this was not investigated further.

The analogy with the situation in the kinetoplast-mitochondrial genome of the trypanosomatids is striking and illustrative of a basic evolutionary dichotomy. In trypanosomatids, a few gRNAs are encoded in the maxicircle DNA, but most are encoded in the catenated minicircle DNA molecules (Pollard et al., 1990; Sturm & Simpson, 1990; Maslov & Simpson, 1992). In *T. borreli*, the identified gRNAs are encoded in 180-kb molecules that do not appear to contain structural genes and represent the homologue of the trypanosomatid minicircle genome. In some trypanosomatids, such as *C. fasciculata* C-1 strain, more than 90% of the minicircles encode a single gRNA and the remaining minor minicircle sequence classes encode all the gRNAs required for editing (Yasuhira & Simpson, 1995). This appears similar to the situation in *T. borreli*, in which a single putative gRNA-encoding sequence (the *Sca* I repeat) is present at 1,000 times the frequency of the individual identified gRNA genes. However, in trypanosomatids, the minicircle molecules are organized into one or more conserved regions containing the origins of replication for both strands and variable regions containing the gRNA genes. In *T. borreli*, the gRNA-containing genomic fragments do not appear to contain a conserved region, which is consistent with the localization of these genes on a large circular molecule that most likely has a single origin of replication.

It is intriguing to speculate that the organization of gRNA genes in *T. borreli* in large circular molecules is an evolutionary precursor of the minicircular type of gRNA gene organization in the trypanosomatids. However, with only a single example, a parsimony argument cannot be applied, and it is equally probable at this point that this is a derived character in the cryptobiid lineage. Further comparative investigations of bodonids and other cryptobiids will be necessary to illuminate this situation.

MATERIALS AND METHODS

Cell culture

The axenic culture of *T. borreli* Pg-JH was maintained in LIT medium supplemented with 10% fetal bovine serum (Gemini Bioproducts, Inc.) at 16 °C as described previously (Maslov & Simpson, 1994).

DNA and RNA isolation

For DNA isolation, cells were lysed with 0.5% SDS, 0.1 mg/mL proteinase K at 60 °C for 3 h in SET solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA). The lysate was extracted twice with phenol/chloroform. DNA and RNA was precipitated by the addition of 1 volume of isopropanol, and dissolved in 10 mM Tris-HCl, 1 mM EDTA. For some purposes, RNA was digested with RNase A. For RNA isolation, a standard guanidium isothiocynate/acid phenol method was used (Chomczynski & Sacchi, 1987).

Construction of gRNA library

Total cellular RNA (100 μg) was electrophoresed on a urea-polyacrylamide gel and 30–70-nt molecules recovered. The gel-purified RNA was C-tailed at the 3′ end with poly[A] polymerase (GIBCO/BRL) and CTP. cDNA was synthesized by priming with S1881-3 (5′-GGGGGGGGGGGGGGG[ACT]-3′), and the cDNA was then 3′-ligated to an "anchor" oligonucleotide, S1859 (5′-*p*-CACGAATTCACTATCGATTCTGG AACCTTCAGAGG-NH_2-3′) (Edwards et al., 1991). The anchor-ligated cDNA was PCR-amplified by using S1879 (5′-AAGGATCCGGGGGGGGGGAAAAA-3′) and the antisense-anchor oligonucleotide, S1408 (GTTCCAGAATCGAT AGTGAATTCGT). This PCR step selects for cDNA species containing oligo[U] sequences at the 3′ ends. PCR products were cloned into pBluescript II SK+ (Stratagene) and randomly selected clones were sequenced.

Southern blot analysis

Total cellular DNA (10 μg) was digested with an appropriate restriction enzyme, electrophoresed on a 0.75% agarose gel, and transferred to Hybond N+ (Amersham). The membrane was hybridized with 5′-labeled oligonucleotide probes specific for each of the gRNA sequences (S2011 for g085, 5′-TTGTTATTTCTTTTTGTGTTGTATA-3′; S2012 for g040, 5′-CTATTCTGCGCGTATTTTGTATGTA-3′; S2041 for g009, 5′-TTACTTGTCTACTTTATTTTGTTTA-3′; S2042 for g034, 5′-TATTTTTTTGGCAGTTTCTCATAGT-3′; S2043 for g043, 5′-TTATCCAGTGATTTATTTCGTGCAT-3′) at 45 °C for 16 h and 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.

5′ RACE of gRNAs

A mixture of a 5′ end-labeled primer (3–5 pmol) complementary to a specific gRNA (see Fig. 2) and total cellular RNA (20 μg) was ethanol-precipitated, dissolved in water, denatured at 65 °C for 15 min, and chilled on ice. Primer extension reactions were performed at the appropriate temperature for 30 min in the presence of 0.5 mM of each dNTP and 200 units of Superscript II reverse transcriptase (GIBCO/BRL). The extension products were purified from denaturing polyacrylamide gel and ligated with an anchor oligonucleotide (S1859). The anchor-ligated cDNA was PCR-amplified with a nested gRNA-specific oligonucleotide (see Fig. 2) and a antisense-anchor oligonucleotide (S1408). PCR products were cloned into the pBluescript II SK+ vector and randomly selected clones were sequenced.

Contour-clamped homogeneous electric field (CHEF) gel electrophoresis and blotting analysis

Agarose blocks with embedded cells were prepared as described elsewhere (Rovai et al., 1992), washed two times with 0.2 M Tris-HCl, pH 8.0, 0.1 M EDTA, and placed in a 1.5-mL microcentrifuge tube with 0.5 mL of the same solution. The blocks were irradiated with gamma radiation from a ^{60}Co source at ambient temperature with a dose rate of 4.6 cGy/s. CHEF gel electrophoresis was conducted with a 1% agarose 0.5× TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.3) gel, using a voltage gradient of 5 V/cm and a 30-s switch time at 10 °C for 40 h. DNA separated on the gel was transferred to Hybond N+ and the filter was probed with a 475-bp PCR-amplified GAPDH gene fragment, a 1-kb *Sca* I repeat unit fragment, a 940-bp Component II fragment (containing the cytochrome oxidase subunit III [COIII] gene), or the 594-bp g009-gRNA coding sequence. Molecular weight markers consisted of lambda DNA ladders (CLONTECH) and *Saccharomyces cerevisiae* chromosomes (CLONTECH) embedded in agarose blocks.

Quantitation of cellular DNA components

Fragments of the GAPDH gene (475 bp) and the COIII gene (940 bp) were PCR amplified using S2104 (5′-CCCGGATCC GCTACTCAGAAGACAGTT-3′) and S2105 (5′-GAGTCTTT GGAATTCATAAA-3′), and S2128 (5′-CCCGAATTCTTATG TATTTGTTTAGAATA-3′) and S2129 (5-CCCGAATTCTAC ATATCCCTTCTGTACT-3′), respectively. The 594-bp *Msp* I fragment that contains the g009-gRNA coding sequence (gg009) was purified from the original plasmid clone. An approximately 1-kb *Sca* I repeat unit sequence was cloned into the *Eco*R V site of the pBluescript II SK+ plasmid, and the three DNA fragments obtained above were also cloned into appropriate restriction sites of the same plasmid. This plasmid construct, termed pREF, in which DNA fragments from four different cellular DNA components were located tandemly in equimolar amounts, was used as a reference DNA for quantitative dot blot analysis. *Sca* I-digested genomic DNA from *T. borreli* and *Hin*d III-digested pREF DNA were serially diluted and spotted on Hybond N+ membrane using the HybriDot Manifold (BRL). Each of four identical blots was hybridized with one of the four DNA fragments that were labeled with the Prime-It II Random Primer Labeling Kit (Stratagene) at 65 °C for 16 h, and washed twice with 2× SSC, 0.1% SDS for 10 min each, once with 1× SSC, 0.1% SDS for 15 min, and once with 0.1× SSC, 0.1% SDS at 65 °C.

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