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Guide RNAs for Transcripts with Developmentally Regulated RNA Editing Are Present in Both Life Cycle Stages of *Trypanosoma brucei*

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RNA editing of several mitochondrial transcripts in *Trypanosoma brucei* is developmentally regulated. The cytochrome *b* and cytochrome oxidase II mRNAs are edited in procyclic-form parasites but are primarily unedited in bloodstream forms. The latter forms lack the mitochondrial respiratory system present in procyclic forms. Editing of the NADH dehydrogenase 7 (ND7) and ND8 transcripts is also developmentally regulated but occurs preferentially in bloodstream forms. Other transcripts, cytochrome oxidase III and ATPase 6, are edited in both life forms. We have identified many minicircle-encoded guide RNAs (gRNAs) for ATPase 6, ND7, and ND8. The characteristics of these gRNAs reveal how extensively edited RNA can be edited in the 3′-to-5′ direction. Northern (RNA) blot and primer extension analyses indicate that gRNAs for transcripts whose editing is developmentally regulated are present in both procyclic and bloodstream form parasites. These results suggest that the developmental regulation of editing in these transcripts is not controlled by the presence or absence of gRNAs.

Trypanosoma brucei is a protozoan parasite which cycles through both a mammalian host and an insect host. During this complex life cycle, the mitochondrial respiratory system is developmentally regulated. Mammalian bloodstream forms rely on glycolysis for their energy production and lack cytochromes and Krebs cycle enzymes, while procyclic (insect) forms contain a fully functional mitochondrion (for reviews, see references 6 and 33). The mitochondrial DNA (mtDNA) of *T. brucei* is composed of two types of circles, a homogeneous population of approximately 50 22-kb maxicircles and thousands of heterogeneous 1-kb minicircles (26). These circles exist in a large catenated network called kinetoplast DNA. The maxicircles are homologous to the mtDNAs of other eukaryotes and encode components of the mitochondrial respiratory system.

Many maxicircle transcripts are encoded in a cryptic form and must undergo RNA editing before they are functional transcripts. This process posttranscriptionally alters the mRNAs by the addition of uridines not encoded in the genome and in some cases by the deletion of encoded uridines (for reviews, see references 27 and 28). For many of the transcripts, the functional initiation and termination codons appear to be created by editing, suggesting that editing plays a crucial role in the regulation of expression of these genes. Indeed, while all mitochondrial genes are transcribed in both procyclic and bloodstream form parasites (11, 20, 30), RNA editing of several transcripts is developmentally regulated. The cytochrome *b* (CYb) and cytochrome oxidase II (COII) mRNAs are edited in procyclic form parasites but are primarily unedited in bloodstream forms (10, 12, 13). Editing of the NADH dehydrogenase 8 (ND8) mRNA is also developmentally regulated, but in this instance editing occurs preferentially in bloodstream forms (24). Other mitochondrial transcripts, such as ATPase 6 (A6) and COIII, are constitutively edited in that they are equally edited in both life forms (2, 7). The editing of ND7 is more complex since there are two editing domains. The 5′ domain is equally edited in both bloodstream and procyclic forms, but editing of the 3′ domain occurs preferentially in bloodstream forms (18).

While the exact mechanism of editing is unknown, its specificity is thought to be provided by small transcripts called guide RNAs (gRNAs), many of which are encoded in minicircles. These gRNAs are complementary (allowing G:U base pairs) to portions of the fully edited sequence. They range in size from approximately 50 to 70 nucleotides (nt) and contain a short region at their 5′ end that can base pair just 3′ of regions to be edited (2, 3, 18, 21, 22, 31). We have identified numerous gRNA transcripts for ND7, ND8, and A6. All of the gRNAs are encoded in minicircle DNA between 18-bp inverted repeat sequences. The characteristics of these gRNAs reveal how extensively edited RNAs can be edited in the 3′-to-5′ direction. Northern (RNA) blot and primer extension analyses indicate that gRNAs for edited mRNA sequences are present in both procyclic and bloodstream forms. These results suggest that developmental regulation of editing of these transcripts is controlled by factors other than the simple presence or absence of gRNAs.

MATERIALS AND METHODS

Parasites, isolation of mitochondria, and RNA extraction. *T. brucei brucei* clone IsTar from stock EATRO 164 was grown and isolated as previously described (29). Bloodstream forms were harvested after 4 days of infection in rats and were virtually all long slender forms. Cells were frozen in liquid N_2 and stored at $-70°C$ prior to RNA extraction or were used immediately for isolation of mitochondria. In general, for both procyclic and bloodstream forms, 0.5×10^{11} to 2.0×10^{11} cells were used for an isolation of mitochondria. Mitochondria were isolated by the method of Harris et al. (14) and stored frozen at $-70°C$ until RNA extraction. mtRNA was isolated by the acid guanidinium-phenol-chloroform method (5).

Oligonucleotide probes and primers. For detection of

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gRNAs, the following oligonucleotides were used:

gND7-506 5′-CACTAACTATACTACAGGTTATTTACATCG-3′
gND7-398 5′-CGTCTATTAATTACAGAATCTACCC-3′
gND7-385 5′-CCTATAATTCTAATAGTTCACTG-3′
gND7-305 5′-GACTCTTTGTCGTTGTTG-3′
gND7-177 5′-CTACACTCACTTAATATACGCACG-3′
gA6-149 5′-CTTTCTATTCGTCTCTTGTTGTGTT-3′
gA6-48 5′-CTTTATACTACAATCTCGCAACTG-3′
gA6-14 5′-CACTGTCAAAATCTGATTCGTTATCGGAG-3′

The gRNA 18-bp inverted repeat primer has a *Bam*HI linker at its 5′ end, and its sequence is 5′-CGG-GAT-CCC-G(AAGT)(AAG)-(AAG)(AAG)T-(AAG)(AAGT)(AAGT)-(AAG)TA-ATA-(AG)AT-A-3′. The proportion of bases at the degenerate sites are indicated within parentheses.

gRNA cloning and sequencing. *T. brucei* minicircle sequences lying within 18-bp inverted repeat sequences were amplified by polymerase chain reaction (PCR), using a degenerate primer based on the 18-bp inverted repeat sequences (see above) (16). Forty-four nanograms of *T. brucei* kinetoplast DNA was amplified in a 100-μl reaction with 50 pmol of primer, 1 U of *Taq* polymerase (AmpliTaq; Cetus), 200 μM each deoxynucleoside triphosphate, 10 mM Tris (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM EDTA. The sample was denatured for 2 min at 92°C and received five cycles of denaturation at 92°C for 30 s followed by 20 s each at 23, 37, 45, 60, and 72°C. The reaction mixture was then subjected to 30 cycles of denaturation at 92°C for 1 min and incubation at 42°C for 30 s and 72°C for 1 min. The amplified product was purified from an agarose gel, *Bam*HI digested, and ligated into the unique *Bam*HI site of Bluescript II SK− (Stratagene). Sequencing was done by the dideoxy-chain termination method, using Sequenase (United States Biochemical) according to the manufacturer's instructions.

Northern blot analysis. Five micrograms of mtRNA isolated from bloodstream and procyclic forms was electrophoresed in 15% acrylamide gels containing 7 M urea and 1× TBE buffer (89 mM Tris-borate, 2.5 mM Na_2EDTA, pH 8.3). Gels were electroblotted at 400 mA overnight onto Nytran filters in TEA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2), using a Hoefer TE42 Transphor electrophoresis unit. Hybridization conditions for ^{32}P-end-labeled oligonucleotide probes were as described previously (10). Washes for gND7-506, gND7-398, gND7-177, and gA6-14 were for 1, 2, and 3 min in 5× SSPE (1× SSPE is 90 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA)–1% sodium dodecyl sulfate (SDS) at room temperature and for 3 min in 1× SSPE–1% SDS at 50°C. Washes for gA6-149 and gA6-48 probes were as described above, using a final wash temperature of 55°C. Blots were stripped by boiling for 3 min in 0.1× SSPE–0.1% SDS prior to reuse.

CYb gRNAs were detected by probing Northern blots of mtRNA with ^{32}P-labeled in vitro transcripts homologous to the 5′ edited portion of the CYb mRNA. Hybridization conditions were 5× SSPE–1% SDS at 42°C for 12 h. Blots were then washed at room temperature for 1, 2, and 3 min in 5× SSPE–1% SDS and for 3 min in 5× SSPE–1% SDS at 42°C.

Primer extension analysis. 5′ ends of gRNAs were determined by primer extension analysis using gel-purified ^{32}P-end-labeled oligonucleotides and procyclic mtRNA. Primers were heated to 85°C for 3 min with 2.5 μg of procyclic mtRNA and hybridized at 42°C for 20 min. The reaction mixtures were allowed to cool to room temperature and were extended with mouse mammary leukemia virus reverse transcriptase (Bethesda Research Laboratories) according to the manufacturer's directions. Products were examined on 6 and 15% polyacrylamide sequencing gels. For determination of transcript levels, gel-purified ^{32}P-end-labeled oligonucleotides were hybridized with 10 μg of bloodstream or procyclic form total RNA and processed as described above. Identical reactions using 10 μg of total RNA from a diskinetoplastic mutant of *T. brucei* were run as controls for primer specificity. Products were run on 15% polyacrylamide sequencing gels, and nonsaturated autoradiograms were analyzed on a Bio-Rad model 620 videodensitometer. Levels of ND7 mRNA transcripts were determined by using oligonucleotide Murf 3.1, which is complementary to the HR3 region near the 5′ end of the transcript (18).

Computer analysis. DNA sequences were compiled and analyzed by using DNASTAR. The DNASTAR Patterns program, modified to allow G:U base pairs, was used to search for gRNA sequences and alignments. For the gRNA search, the entire fully edited ND7, ND8, and A6 transcripts were divided into blocks of 20 nt with a 10-nt overlap. The search parameters allowed a single mismatch in the 20-nt block with no gaps.

RESULTS

gRNA identification. A total of 18 gRNAs have now been identified for the extensively edited ND7, ND8, and A6 transcripts. All of these gRNAs are encoded in minicircle DNA between 18-bp inverted repeats (Fig. 1). All 12 ND7 and A6 gRNAs are aligned with the edited mRNAs in Fig. 2 and 3; they are named to indicate the mRNA and the furthest 3′ editing site complementary to the gRNA. Editing sites are defined as the sites between nonuridine nucleotides (19). The alignment of the ND8 gRNAs with their mature message has been previously reported (24). gA6-14 (previously gA6-1 [2]) and gA6-48 are encoded by the H40 minicircle (17), which also encodes a COIII gRNA (22), accounting for all three sequences bounded by 18-bp inverted repeats in this minicircle. The P2 minicircle (17) encodes gND7-506 and gND7-398 (previously gND7-1 and gND7-2 [18]), while the P1 (17) and B10 (25) minicircles encode gA6-149 and gND8-205, respectively. gRNAs gA6-223 and gND7-305 are encoded in the minicircles of different *Trypanosoma equiperdum* strains (1, 21). The occurrence of a gRNA like gND7-305 in *T. brucei* was confirmed by primer extension analysis (data not shown). The other gRNAs were obtained by PCR amplification of minicircle cassettes (see Materials and Methods), except for gND7-550, which was cloned as a gRNA-mRNA chimeric molecule (19). The gene encoding this gRNA transcript has not been identified; therefore, the flanking sequence 18-bp repeats are not known. The gRNAs are complementary to various regions of the edited mRNAs, suggesting that multiple gRNAs direct the editing of extensively edited transcripts.

gRNA characteristics. The gRNA coding sequences have between 39 and 52 nt of complementarity with mature RNA, depending on the particular gRNA. The regions of complementarity start 33 to 39 nt downstream of the 18-bp inverted repeats. The 5′ ends of six gRNAs, gND7-506, gND7-398, gND7-385, gND7-305, gND7-177, and gA6-48, were determined by primer extension analysis (Fig. 1). In addition, the 5′ end of gND7-550 was obtained by anchor PCR cDNA cloning (19). Most of the gRNAs have the 5′-RYAYA-3′ sequence which has been proposed as a transcription initiation site (21). Two gRNAs however, do not follow this

ND7 gRNAs

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gND7-550   ATATATAAatgcagtggaTTAAGTGTAAGATGATATAAATGTGAATATTTTTTTTTT
gND7-506   GAAATATGATAATAGATATGTATGTAGGAATTAGATAATTATAGATATAAAtacgatgtaaatAACCTGTAGTATAGTTAGTGTATATAGTGAAATTTATAAGTTAATTAATATTATGATTATATATTTATTATTATATATTTT
gND7-398   GAAATAATGTAATAGATAGGTAGATAGTAATAATATAATTTAAATTGTATATAtattaaaactaTGGGTAGATTCTGTAATTAATAGACGTGTAAAATTAGTTATTATATATTAAAGATAATCTATTTATTATTTAATTTT
gND7-385   TAAATAAAATAATAGATAGAGAATTATTTAGATTTATATATATAAGCGAAATTACAatacatgatataTACAGTGAACTATTAGAATTATAGGTAATAAGATTTATAAATTAACTTATTATGATTTATTTATTATTTTATCTT
gND7-305   AAAGTGATGTAATAGATAGAAACATAATAATTTAGTTTGGTAGTATATACAtatcaacaacGACAAAGAGTCAGTGAAATTAGAGATAAAGTTATTGTGTAGTTATATAATTAAATTAATCTATCTATTATTTATTTCT
gND7-177A  AAAATATTATAATAAATAGGATGGAGTAATATTATATAGATGTATATAGTAtacaaatcaacaaaTTCGTGCGTATATTAAGTGGGTGTAGAAGTGAATGATTTAGTTTATTTATAGTTATAGTCTATTTATTATTTCATTTA
gND7-177B  GGAATAAAGTAATAAATAGGATGGAGTAATATTATATAGATGTATATAGTAtacaaatcaacaaaTTCGTGCGTATATTAAGTGAGTGTAGAAGTGAATGATTTAGTTTATTTATAGTTATAGTCTATTTATTATATTTTT
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A6 gRNAs

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gA6-223    GAAATAGGATAATAAATAGATGCTTAATGTAGTAGTATAATTAATATCGTATATaacacaaaccaaTGAACAAGTATAAGTCAAATAGATGAGATAATTTAGTGTATTATAATGAAGATCTTATTATTTATTTATTATTTTATTGA
gA6-149    GAAATAATATAATAGATAATGATAATATAATATTAATTTAATAATCACTATATATaacacaacaaGAGACGAATAGAAAAGTTATCTGTGAAATTATAGTTACTTATATGATATTGTACTATTTATCTATTATAAATATTTT
gA6-92     AAAATAATATAATAGATATGATAATAATTAATTTATATGATATAATGTATaaacaaaacacaaaaataaGTAAAGTAGGTCAGTGATAAGATATACAGTTAATAATCTATATATGTTAATTGTTATATTTATTTATTATCTCACTTA
gA6-51     GAAATGTGATAATAGATAAGGTATAAGGTAATTAAATGATAAAATTATATATAAattatcaactgTGAAATTGTAGTACGAGAAATAAGCAATGTTAATTATAATATTATTGATTAATTTGTATTTATTACATTATTTC
gA6-48     AAAATAAAGTAATAGATAGGATGAATAATAATTAATATGATGATTATGATATATaatccattatcaGTTGCGAGATTGTAGTATAAAGAATAATTAATAGTTAAATTTAATATAAGACTAATATTTATTTATTATAAGATTTT
gA6-14     AAAATGATATAATAGATATGTAATGATAGAATTAATGTATGAAATTAATATATACtataactccgaTAACGAATCAGATTTTGACAGTGATATGATAATTATATATAATGTTATTGAATAATCTATTTATTATAAATATTTC
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ND8 gRNAs

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gND8-205   AAAATAGAGTAATAGATAGATGGATAATATAAGTTATATATGAATTATAATATacaatggtaactccGATGGGTGGGTAATTAGTGATATGATGAATTTAATACTATGATTTATTATTTATTTATTATT
gND8-190   TAAATAAGATAATAAATAGGAATATTAGTTAAATTTAAATGATAATAAATATatataaacgcaataaatggttatCATGAGTCAATGAATTAATGATATTTAGTTATATAATTATTGTAGTTATATTTATTATTTCATTTA
gND8-164   GAGGTAATATAATAAATAATGTTAATAGTTAATAGTTATATGATACAATATATAtaaatcacatatacgGCAAGTTATATAGCGTTGTAAATGATGTCAATTATATATATGAATTATTAAATCTATATTTATTATTTTATTTA
gND8-47    AAAATATAATAATAGATAGAGTGATAAAATGATATGAATGAATATTATATATATtaaaacaacaaTCAAATGATAGAAGCTTGAGTGACTGTGGATATAAGATATTAAATTAAATGATTAATTAATTCCTTATTTATTATATTATTTT
gND8-34    TAAGTGAAGTAATAAATAGAAGTATTAATAATATAGATATATAATATAGATATataactacacaGAACATAGATAAGAGTCAGATAGTATAAAGTGATATATTAGTTATAATTACATATTAATACTTATTTATTATTTTATTTT
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FIG. 1. Minicircle gene sequences for ND7, A6, and ND8 gRNAs. The 18-bp minicircle cassette sequences which have been identified as potential ND7, A6, and ND8 gRNAs are shown. The 18-bp inverted repeat sequences flanking the gRNA cassette are underlined. The 5′ end of the gRNA transcript as determined by primer extension analysis or anchor PCR cDNA cloning is double underlined. The region complementary to the fully edited mRNA sequence is shaded, and the predicted 5′ anchor sequences are shown in lowercase.

pattern. For both gND7-506 and gND7-385, the transcript appears to start at a triple A located 31 nt downstream of the 5′ 18-bp repeat (Fig. 1). It is interesting to note that the 5′ end of gND7-305 determined in *T. brucei* is identical to that determined in *T. equiperdum* (21).

The gRNA coding sequences described here have between 7 and 19 nt at their 5′ ends that can form predominantly Watson-Crick base pairs (anchor) with the mature transcripts, implying that the gRNA 5′ end functions in mRNA selection (3) (Fig. 1). Inclusion of one G:U base pair increases the anchor sequence of gND7-506 from 4 to 12 nt (Fig. 2). Only gA6-14, gND7-506, and gND7-550 can form an anchor duplex with unedited RNA (Fig. 2 and 3). gA6-14 and gND7-506 are complementary to sequences at the 3′ ends of editing domains, and gND7-550 can form a duplex with a stretch of 10 nt (with a single mismatch) which is unedited in the mature message. The other gRNAs require editing to create the mRNA sequence that is complementary to the anchor. This finding suggests that some gRNAs initiate editing and create sequences that are used by other gRNAs. Some of the identified gRNAs are indeed complementary to overlapping stretches of sequence. gND7-398 overlaps the last 12 nt specified by gND7-385 (Fig. 2). Similarly, gND8-47 overlaps the last 10 nt specified by gND8-34 (24). Thus, editing directed by one gRNA creating the sequence used by another could determine the order of gRNA use and a general 3′-to-5′ progression of editing. In addition, gRNAs for extensively overlapping regions were identified. For example, gND7-177A and -B are identical in the region of mRNA complementarity except for one G-A transition (Fig. 2). gA6-51 and gA6-48 specify the mature sequence for essentially the same region, with gA6-51 anchoring 7 nt 5′ of gA6-48. These gRNAs differ only in the relative positions of G:U pairing sites (Fig. 3).

gRNA transcript analyses. Northern blots of RNA extracted from isolated mitochondria reveal some transcript size heterogeneity (sizes range from 45 to 60 nt) with probes for six of the gRNAs discussed above. gRNAs appear to have specific 5′ ends with variable lengths of posttranscriptionally added 3′ poly(U) tails (4, 21). Such variable 3′ uridine addition is presumably responsible for the difference between the coding sequence and transcript size and for the size heterogeneity of up to 18 nt seen in Northern blots. All of the gRNAs examined are present in both bloodstream and procyclic form RNAs. For most of the gRNAs, a greater length heterogeneity and a greater proportion of smaller sizes is observed in the bloodstream forms. Interestingly, gND7-398 transcripts (Fig. 4B) are present in procyclic forms, although little or no editing of the corresponding region of the mRNA is observed in that stage of the life cycle (18). Similarly, transcripts for ND8 gRNAs are also present in both life cycle forms, although editing is limited to the bloodstream forms (24). To determine whether gRNAs are present in both life stages for transcripts which are predominantly edited in procyclic forms, the Northern blots of mtRNA were probed for CYb gRNAs. Editing in CYb is limited to the 5′ end of the transcript and involves an addition of 34 uridines (10). Because the gRNAs for the *T. brucei* CYb transcript have not been identified, the blots were probed with ^{32}P-labeled in vitro transcripts homologous to the 5′ edited mRNA of CYb (Fig. 4C). This analysis detected gRNA-size transcripts of approximately 50 nt in both bloodstream and procyclic form mtRNAs.

To confirm the presence of gRNA transcripts in the different life forms, primer extension analyses using bloodstream and procyclic form total RNAs were done for three ND7 gRNAs, gND7-398, gND7-385, and gND7-305. These gRNAs are located in the 3′ developmentally regulated editing domain of ND7. Relative transcript levels were determined by running the primer extension products on

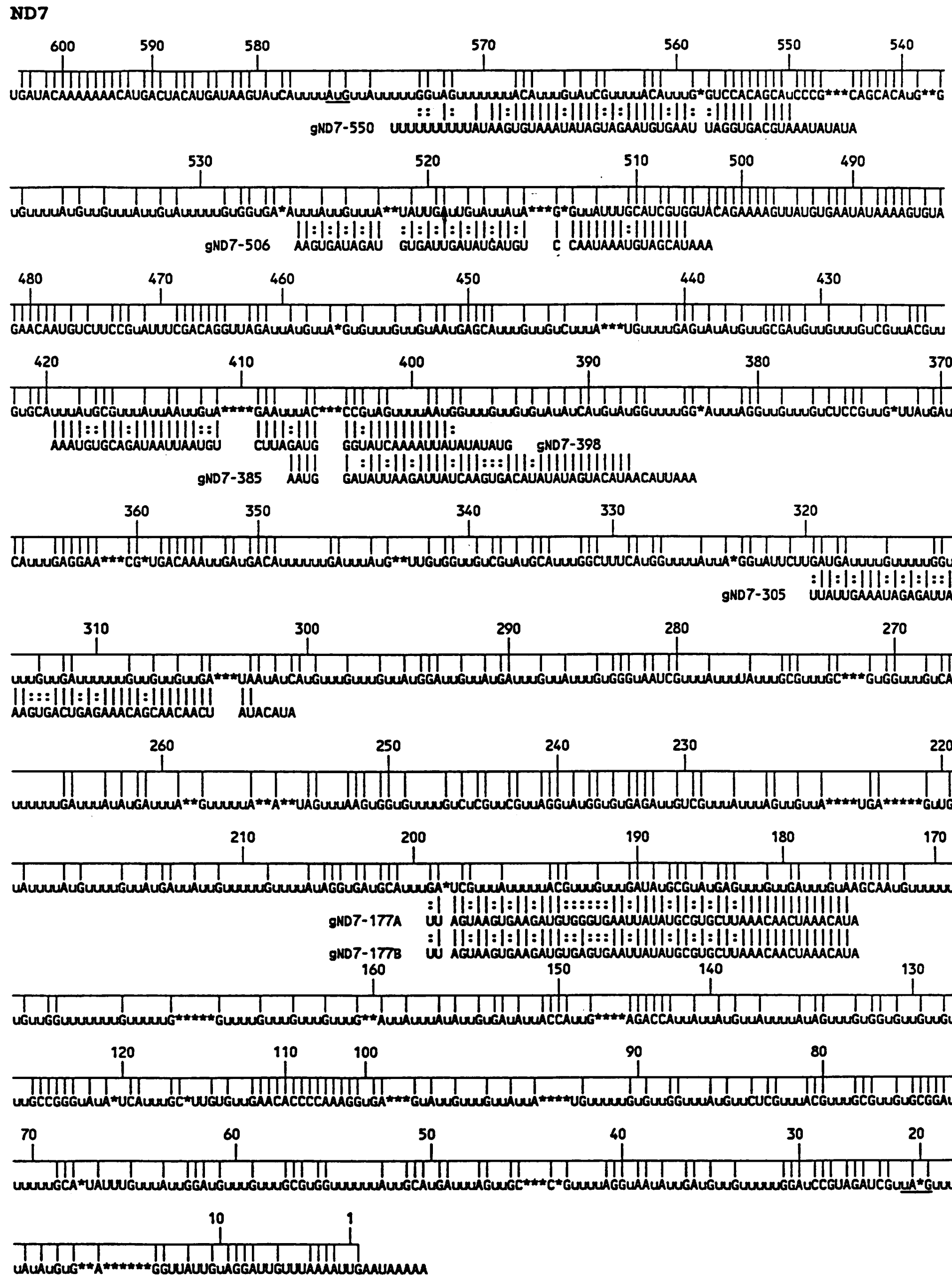

FIG. 2. Alignment of the potential ND7 gRNAs with the fully edited mRNA. The sequence of the fully edited ND7 transcript is presented, with the gRNA sequences aligned beneath the sequence; complementarity is indicated for G:U basepairing (:) and for Watson-Crick base pairing (|). Asterisks in the transcript sequence mark encoded uridines that are absent from the RNA sequence. Potential editing sites, the sites between nonuridine nucleotides, are numbered 3′ to 5′ from the most 3′ non-U or A in the mature transcript.

15% acrylamide sequencing gels and scanning nonsaturated autoradiograms with a densitometer. For all three gRNAs, transcripts were readily detected in both bloodstream and procyclic form parasites. Transcript levels were compared with the relative ratio of ND7 mRNA transcripts found in the different life forms (Table 1). While the relative ratios of gRNA to mRNA transcript levels are similar for the different life forms, two of the gRNAs, gND7-385 and gND7-305, had gRNA-to-mRNA ratios which were lower in the procyclic forms.

DISCUSSION

The information specifying the numbers and sites of uridines to be added or deleted in kinetoplast RNA editing appears to be contained in small transcripts called gRNAs, which are complementary to regions of edited RNA (3). In *T. brucei*, all of the gRNAs identified to date are encoded in minicircle DNA between 18-bp inverted repeats, although some may be encoded in the maxicircle. The minicircles contain three and sometimes four sets of 18-bp repeat units

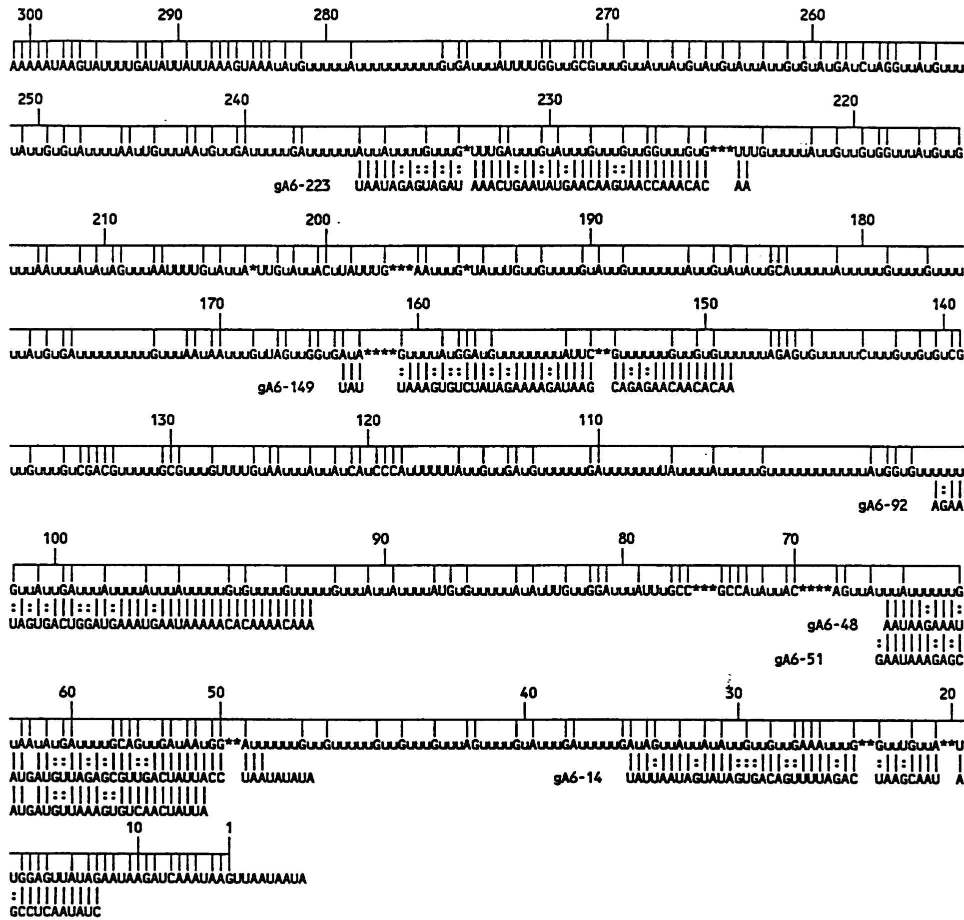

FIG. 3. Alignment of the potential A6 gRNAs with the fully edited mRNA. The sequence of the fully edited A6 transcript is presented, with the identified A6 gRNA sequences aligned beneath the sequence as in Fig. 2.

and hence the potential to encode three or four gRNAs. The presence of the 18-bp sequences as imperfect inverted repeats suggest that they may function (or have functions) in recombination among minicircles, allowing gRNA sequence amplification and diversification. The locations of the 18-bp sequences upstream of the gRNAs suggest that they may play a role in minicircle transcription. gRNA transcripts can be capped in vitro, which suggests that each 18-bp cassette may function as a discrete transcription unit (21, 22). While the 5′ ends of most of the gRNAs have a 5′-RYAYA-3′ sequence, the 5′ ends of two gRNAs, gND7-506 and gND7-385, did not fit this pattern. Both of these gRNAs start with a triple A located 31 bp from the 5′ 18-bp repeat. The 5′ ends of all of the gRNAs determined to date start between 29 to 33 nt from the 18-bp repeat (21, 22). These data suggest that if the gRNA transcription units are functional, initiation may be dependent more on spacing from the inverted repeat than on a consensus RYAYA sequence.

The region of the gRNA which is complementary to the mature message usually begins 33 to 39 bp from the 18-bp repeat. The 5′ portion of this complementarity is predominantly Watson-Crick base pairing and may form the anchor duplex for the initial recognition of the mRNA. The rest of the gRNA shows both Watson-Crick and G:U base pairing with the mature mRNA. The characteristics of the gRNAs described here are very similar to those previously described in *T. brucei* and the other kinetoplastids (3, 21, 22, 32).

The overall 3′-to-5′ progression of editing is implied by the characteristics of the gRNAs, and it suggests that anchor duplex formation between the gRNA and the mRNA initiates gRNA utilization (3, 19). Two of the gRNAs identified here, gA6-14 and gND7-506, can form such a duplex with unedited RNA. Both of these gRNAs correspond to the 3′ limit of an editing domain and thus could initiate editing. In addition, gND7-550 can anchor to a short stretch of nucleotides that is unedited in the mature RNA and may also be able to initiate editing. The other gRNAs can duplex with RNA only after the region corresponding to the gRNA anchor is edited. Many of the gRNAs identified are complementary to overlapping regions of the mRNA, and editing specified by the more 3′ gRNA would create the edited sequence that can form a duplex with the anchor sequence of the next gRNA. Hence, extensively edited RNAs appear to be edited generally in the 3′-to-5′ direction by the sequential utilization of several gRNAs that is controlled by creation of edited sequences capable of duplexing with gRNA anchors. However, the complementarity of some gRNAs with inter-

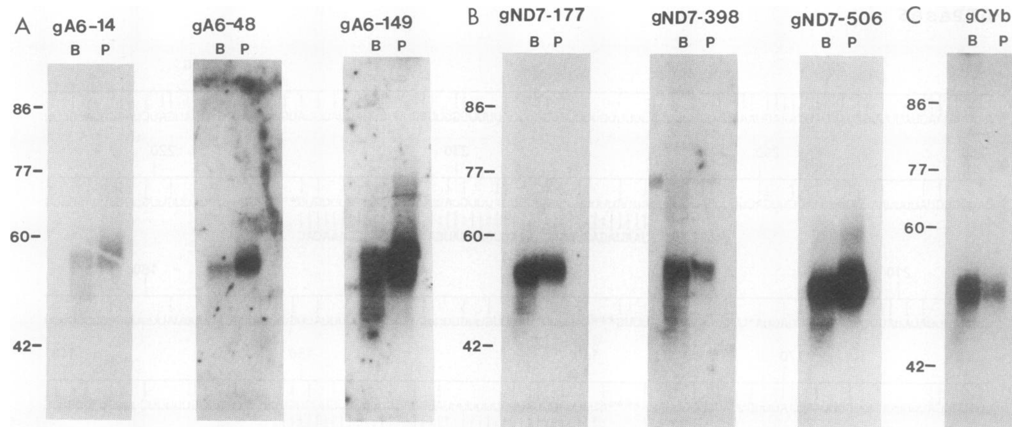

FIG. 4. Northern blot analysis of gRNA transcripts in *T. brucei* mtRNA. Samples (5 μg) of mtRNA from bloodstream form (lanes B) and procyclic form (lanes P) parasites were run on a 15% denaturing acrylamide gel and electroblotted. (A) Northern blots probed with oligonucleotides complementary to gA6-14, gA6-48, and gA6-149 gRNAs; (B) Northern blots probed with oligonucleotides complementary to gND7-177, gND7-398, and gND7-506 gRNAs; (C) Northern blot probed with a CYb in vitro-labeled transcript homologous to the 5′ edited region of CYb mRNA.

nal unedited sequences implies that there may be some flexibility in the order of gRNA usage.

Some of the identified gRNAs overlap extensively. The gA6-48 and gA6-51 gRNAs differ only at G:U base pair positions and provide the sequence information for the same editing sites. gND7-177A and -B differ at a single G:U position; otherwise, the two gRNAs are essentially identical. Such extensively overlapping gRNAs have also been described for COIII, for which it has been suggested that extensively overlapping gRNAs may reflect inefficiencies in the editing process and provide a type of proofreading system (22). Both gA6-48 and gA6-51 have a single G:A base pair mismatch with the mature sequence (Fig. 3). gA6-48 has a perfect match to three partially edited A6 cDNAs, indicating that this gRNA can generate an edited sequence that differs from the mature sequence (19, 31). Several potential gRNAs that are not fully complementary to their respective edited transcript segments have been identified for *T. brucei* (24) and *Crithidia fasciculata* (32) transcripts. The parasites may tolerate such gRNAs by having several gRNAs that can specify overlapping sequences, thus providing a proofreading function. The gRNA mismatches with edited RNA may also indicate that the editing mechanism might allow noncanonical base pairing or not always require a perfect duplex between gRNA and mRNA (19, 32).

Northern blot analyses indicate that gRNAs for ND7, ND8, A6, and CYb are present in both procyclic- and bloodstream-form parasites. Of these four transcripts, one (A6) is constitutively edited (2), two (ND7 and ND8) are edited preferentially in bloodstream forms (18, 24), and one (CYb) is preferentially edited in procyclic forms (10, 13). The presence of ND7 gRNAs in both life cycle forms was confirmed by primer extension analyses, and these data suggest that the relative ratios of gRNAs to mRNAs in the different stages are similar. However, of the three gRNAs investigated, two did appear to be slightly less abundant relative to mRNA in procyclic forms. For both of these gRNAs, the relative ratio of gRNA to mRNA transcripts in procyclic forms was approximately 70% of that seen in bloodstream forms. The significance of this observation cannot be determined until an extensive investigation of the relative gRNA-to-mRNA ratios for many different transcripts is done.

These results suggest that the regulation of editing of mRNAs is not controlled by the regulation of the presence or absence of gRNAs. However, the studies are complicated by the large number and the interdependence of the gRNAs required. Most gRNAs can duplex with the mRNA only after the region corresponding to the gRNA anchor is edited by the downstream gRNA. Therefore, the absence of a single gRNA in the cascade could disrupt the editing process. Because all of the gRNAs for ND7 or ND8 have not been identified, this possibility has not been ruled out. However, the identification of gRNAs in both bloodstream and procyclic forms for CYb, for which the editing is limited to 34 uridine additions near the 5′ end, makes this possibility appear less likely. In *Leishmania tarentolae*, editing of the CYb transcript involves a total of two gRNAs (3). We expect a similar number to be involved in the editing of *T. brucei* CYb.

While the gRNAs for the differentially edited transcripts are present during both life stages, it may be that their specific affinities for the mRNA differ between the stages. The chemical affinity of a reaction is dependent on both the concentration and specific affinities of the reacting species as well as on the temperature. The two life forms of *T. brucei* grow at different temperatures: the insect stage grows at 27°C, and the bloodstream stage grows at 37°C. In addition,

TABLE 1. Ratios of transcript levels of ND7 mRNA and three ND7 gRNAs in bloodstream versus procyclic total RNA[a]

Transcript	BS/P[b]	ΔmRNA/ΔgRNA[c]
ND7 mRNA	0.94	1
gND7-398	0.92	1.02
gND7-385	1.27	0.74
gND7-305	1.29	0.73

[a] Transcript levels in 10 μg of total bloodstream and procyclic form RNAs were determined by primer extension. Products were run on 15% polyacrylamide sequencing gels, and nonsaturated autoradiograms were analyzed by densitometry.

[b] Ratio of optical density (OD) values for bloodstream (BS) versus procyclic (P) transcripts.

[c] ΔmRNA = OD BS RNA/OD P RNA; ΔgRNA = OD BS RNA/OD P RNA.

while mRNA transcripts are found in both life stages, their abundance does appear to be differentially controlled (8, 9, 11, 15, 20), and we have shown that the relative gRNA-to-mRNA ratios can differ between the different life forms. It may be that differences in the specific affinities between the gRNAs and the editing complex at the different life stage temperatures, combined with differences in the ratios of gRNA to mRNA transcripts, may combine to provide a finely tuned mechanism for the regulation of editing in specific transcripts.

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