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Trypanosoma brucei minicircles encode multiple guide RNAs which can direct editing of extensively overlapping sequences

Robert A.Corell[1], Jean E.Feagin[1,2], George R.Riley[1], Tricia Strickland[1], Jeffrey A.Guderian[1], Peter J.Myler[1,2] and Kenneth Stuart[1,2]*

[1]Seattle Biomedical Research Institute, 4 Nickerson Street, Seattle, WA 98109-1651 and [2]Department of Pathobiology, University of Washington, SC-38, Seattle, WA 98195, USA

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ABSTRACT

Small guide RNAs (gRNAs) may direct RNA editing in kinetoplastid mitochondria. We have characterized multiple gRNA genes from *Trypanosoma brucei* (EATRO 164), that can specify up to 30% of the editing of the COIII, ND7, ND8, and A6 mRNAs and we have also found that the non-translated region of edited COIII mRNA of strain (EATRO 164) differs from that of another strain. Several of the gRNAs specify overlapping regions of the same mRNA often specifying sequence beyond that required for an anchor duplex with the next gRNA. Some gRNAs have different sequence but specify identical editing of the same region of mRNA. These data indicate a complex gRNA population and consequent complex pattern of editing in *T.brucei*.

INTRODUCTION

The mitochondrial genome of kinetoplastids is composed of two classes of circular DNAs catenated into a complex network. The maxicircles (~22 kb in *Trypanosoma brucei*) are analogous to the genomic DNA of other mitochondria since they contain genes encoding rRNAs as well as various components of the mitochondrial respiratory system. Many of the primary transcripts for maxicircle genes do not contain open reading frames and undergo RNA editing to produce functional mRNAs (for recent reviews see 1,2). The second component, minicircles (~1 kb in *T.brucei*), contain no protein coding genes, but encode small RNAs.

RNA editing is the post-transcriptional insertion, and less frequent deletion, of uridines in the mRNA (1). RNA editing creates and extends open reading frames, as well as initiation and termination codons (3–7). The information that specifies the edited mRNA sequence probably resides in small RNAs called guide RNAs (gRNAs) which are complementary to portions of the final mRNA sequence by a combination of G·U and Watson–Crick base pairing (8–10). The 5′ end of most gRNAs predicts anchor duplexes of 6–10 bp with mRNAs that are almost exclusively Watson–Crick base-pairs (8,9,11,12). In *T.brucei*, the gRNA genes are usually encoded in minicircle 'cassettes' which are defined by flanking 18 bp inverted repeats (13). Each minicircle can encode three or four gRNAs (12–14; Corell *et al.*, manuscript in prep.).

We have characterized multiple *T.brucei* minicircle-encoded gRNA genes for cytochrome *c* oxidase subunit III (COIII), subunits 7 (ND7) and 8 (ND8) of NADH dehydrogenase (or NADH ubiquinone oxidoreductase), and ATPase subunit 6 (A6). Several of the gRNAs specify edited sequences which overlap on the same mRNAs. In contrast to *Leishmania tarentolae*, where the gRNAs specify edited sequences with a minimum of overlap (15), the *T.brucei* gRNAs often specify edited sequences which overlap substantially more than required to create an anchor duplex for the next gRNA. In some cases two gRNAs direct editing of exactly the same region of the mRNA. Although these analogous gRNA pairs have different sequences, G·U base pairing allows both to specify the same edited sequence. In addition to these overlapping gRNAs, we have found numerous gRNAs which have one or two mismatches with the corresponding mRNA. These data indicate that the gRNA population in *T.brucei* is complex with a gRNA redundancy not evident in *L.tarentolae*. The evolutionary implications of this gRNA complexity and the concomitant minicircle complexity are discussed.

MATERIALS AND METHODS

Organisms and nucleic acids

Procyclic (PF) and bloodstream forms (BF) *T.brucei* (EATRO 164) were cultured as previously described (16). Total RNA was prepared by the method of (17) and kDNA was isolated as previously described (4). The following oligonucleotide primers were used in this study; the underlined portion represents a restriction site linker.

COIII-5: 5′-TGCAAACTCTCATAACTGGTTTCAC –3′
COIII-8: 5′-CACAAAAATCAAATAAACCAGG–3′
COIII-14: 5′-GGTTATTGAGGATTGTTTAAAATTG–3′
$BamdG_{10}$: 5′-<u>GCGGATCC</u>GGGGGGGGGG

* To whom correspondence should be addressed

Cloning

The accession numbers of the edited mRNA sequences of ND7, ND8, and A6 and the edited 3′ portion of COIII are M35536, M63820, M33228, and M20379, respectively. The remaining 5′ portion of the edited mRNA was cloned as cDNA. First strand cDNA synthesis was made from total PF and BF RNA using COIII-8 primer with M-MLV reverse transcriptase (Gibco-BRL). The cDNA was amplified with COIII-8 and COIII-14, an oligonucleotide predicted to be near the 5′ end of the transcript, under the following conditions: one min. denaturation at 94°C, one min. annealing at 45°C, and two min. extension at 72°C for 25 cycles. The PCR product was recovered from an agarose gel, and cloned into the Sma I site of pBluescript II SK⁻. Six BF clones and seven PF clones were picked for subsequent sequence analysis. Anchor PCR (18,19) was used to verify the 5′ terminal sequence. Briefly, cDNA synthesis from total RNA from PF and BF was primed with COIII-5 and the products tailed with dCTP using terminal deoxynucleotidyl transferase (3). The C-tailed cDNA was amplified using COIII-8 and BamdG_{10} by 25 cycles of 30 sec. denaturation at 94°C, 30 sec. annealing at 40°C, and two min. extension at 72°C. The amplified products cloned into the Sma I site of pBluescript II SK⁻ as described above. Five clones were obtained from PF RNA and three from BF. gRNA gene cassettes were cloned by PCR amplification from kDNA with degenerate primers to the 18 base pair repeats as previously described (20).

Minicircle clones were obtained as previously described (20) or by complete digestion of kDNA with *Taq* I. The ~1 kb linear DNA was gel purified using NA45 paper (Schleicher and

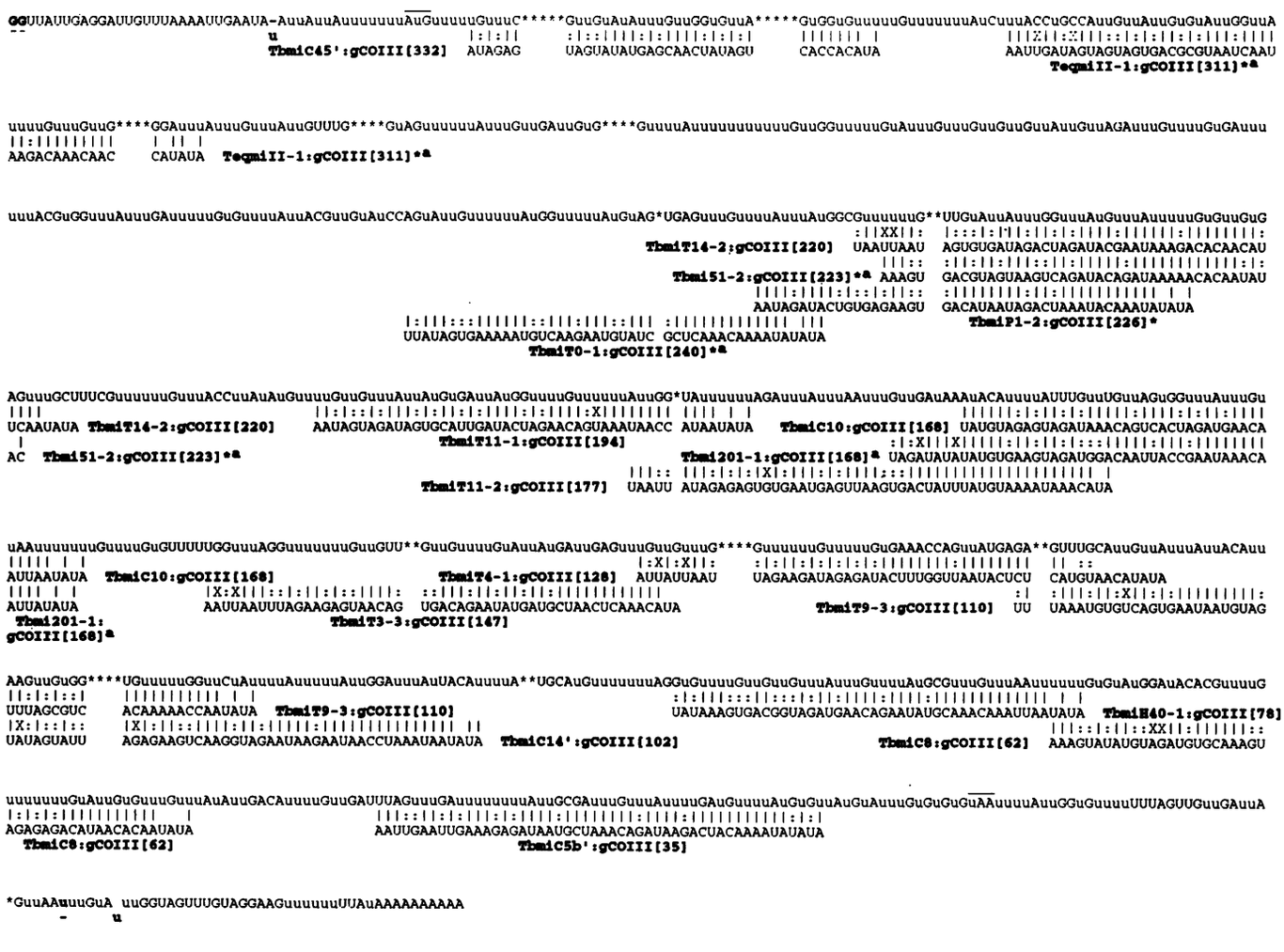

Figure 1. Alignment of gRNAs with edited COIII mRNA sequence. mRNA from EATRO 164 is shown with differences between strain EVE 10 indicated by bold letters. The initiation and termination codons are overlined. Uridines added by editing are in lower case, deletions are designated with an asterisk. gRNAs predicted from minicircle sequences are shown below the corresponding mRNA sequence. The vertical bars indicate Watson–Crick base pairs; colons indicate G·U base pairs, and mismatches are indicated with an X. The 5′ ends of the gRNAs are defined by the conserved RYAYA sequence (11). The 3′ ends of the gRNAs are defined as being immediately upstream of three consecutive mismatches not followed by at least five consecutive matches. gRNA nomenclature: Tbmi indicates sequence of a minicircle from *T.brucei*, TbmiC indicates a cassette clone from *T.brucei* (20), TeqmiI indicates the sequence of a minicircle sequence from the Pasteur strain of *T. equiperdum* (24) and TeqmiII indicates sequence from the ATCC30019 strain of *T. equiperdum* (11). These sequences are also found in the minicircle from *T. evansi* (26,27). This is followed by a clone designation and a number following the dash to indicate the cassette number from the minicircle conserved sequence. The parenthetical designation indicates a gRNA (g) for a cognate mRNA (e.g. COIII) and the editing site. An asterisk (*) indicates a gRNA whose existence in RNA has been previously demonstrated by Northern analysis or cDNA cloning. Note however, that Northern probing of gND7[177] would not have distinguished among the three gRNA sequences shown. A superscripted a ([a]) indicates a gRNA sequence from a strain other than EATRO 164. The complete nucleotide sequence of the edited COIII mRNA was submitted to the GenBank database with Accession number M20379.

Schuell), ligated into the *Cla*I site of pBluescript II SK$^-$, and transformed into DH5α competent cells (BRL). Ten *Taq*áI clones were randomly selected for sequence analysis, in addition to a previously unsequenced *Bam*HI minicircle clone (21).

Sequence analysis

RNA sequencing of the 5′ portion of the *T.brucei* COIII mRNA was carried out as previously described (5,22) using COIII-8 and

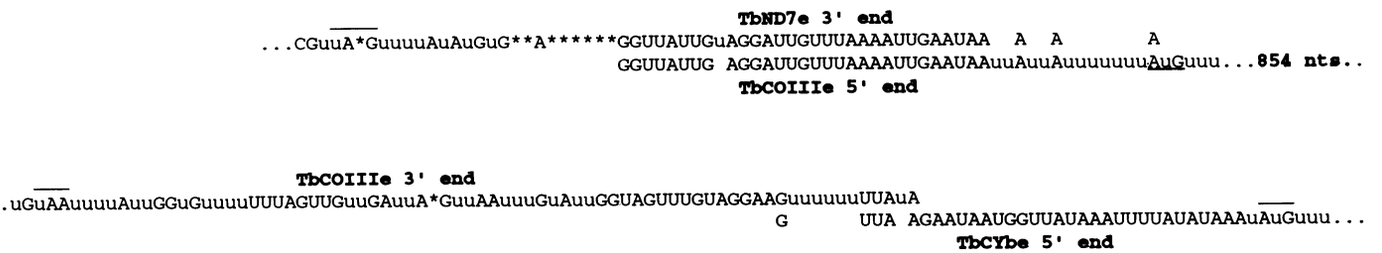

Figure 2. Overlap of the edited COIII, ND7, and CYb genes. Initiation and termination codons are designated by overlining. Other symbols as in Fig. 1.

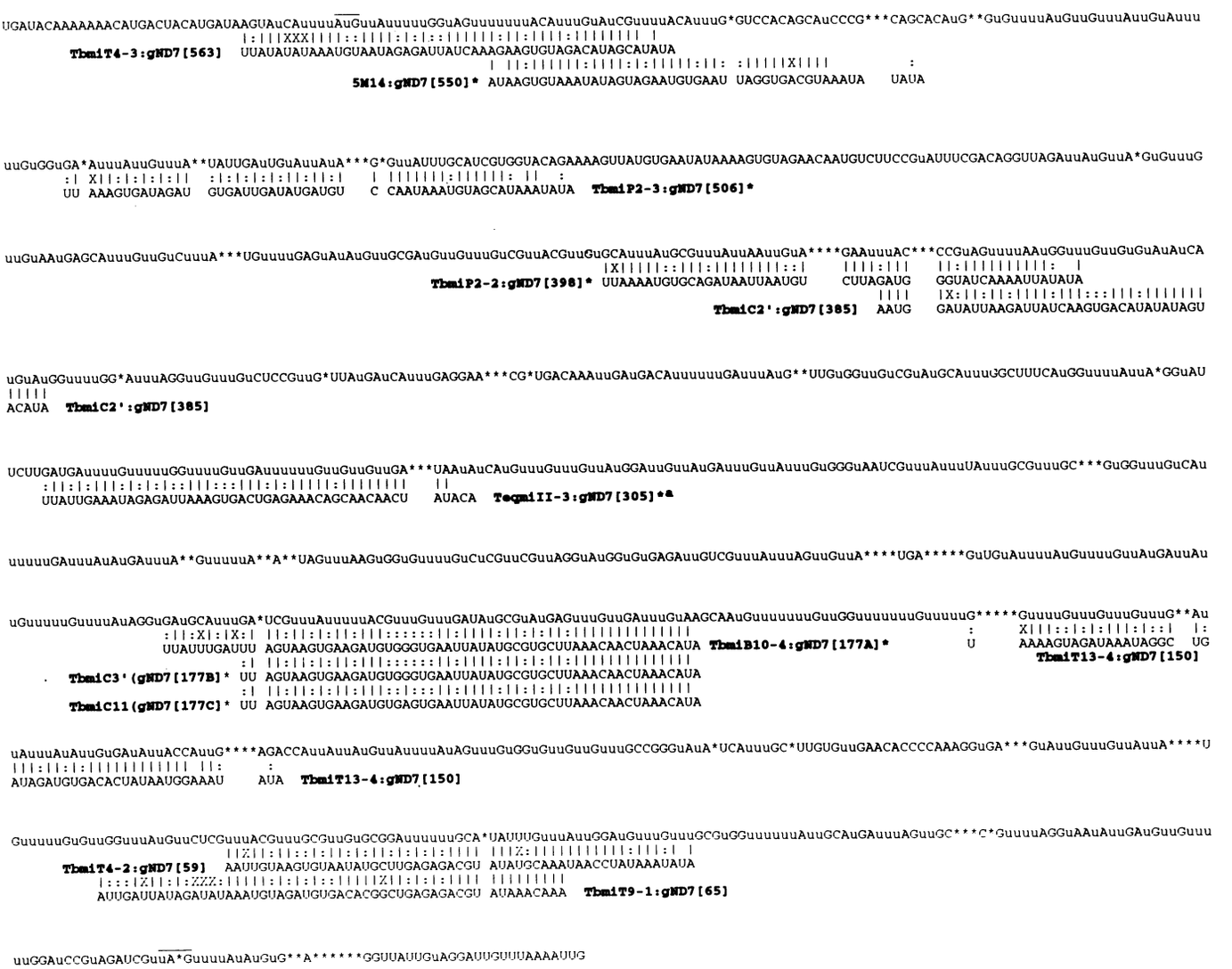

Figure 3. Alignment of gRNAs with edited ND7 mRNA sequence. Other symbols as in Fig. 1.

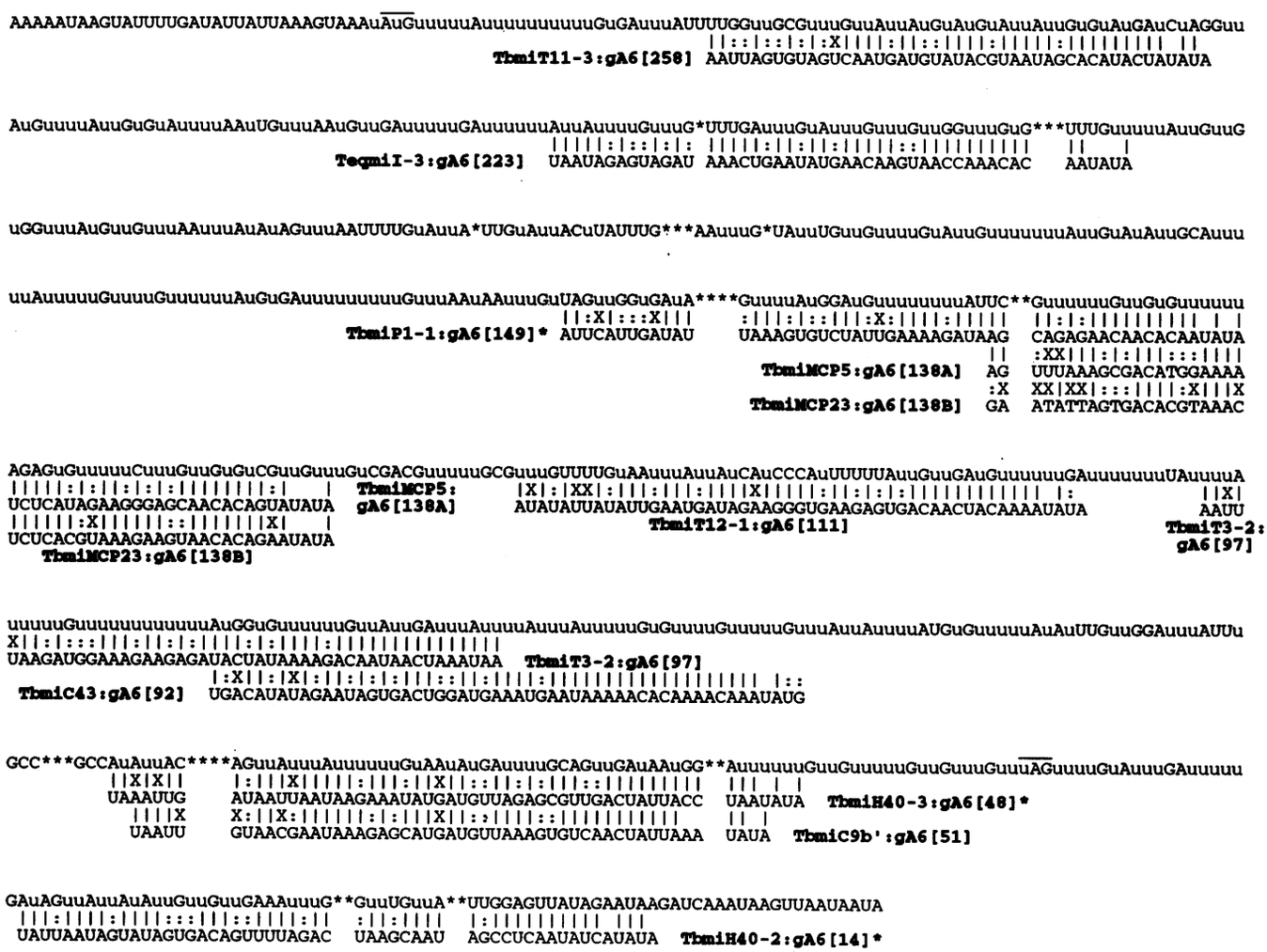

Figure 4. Alignment of gRNAs with edited A6 mRNA sequence. Other symbols as in Fig. 1.

COIII-16 primers. COIII PCR clones were sequenced using the Sequenase double stranded sequencing kit (USB). *Taq* I minicircle clones were sequenced using a Taq Dye Primer Sequencing Kit and a model 373A DNA sequencer (Applied Biosystems, Inc.). Subclones generated by Exo III deletion using the Erase-A-Base system (Promega) were used to obtain complete sequence of the minicircles. Computer analysis of nucleotide sequences was performed using DNASTAR (DNASTAR Inc., Madison, WI). The gRNA genes were identified by computer searches as previously described (20). The sequences of the complete minicircle clones will be published elsewhere.

RESULTS

Complete sequence of edited COIII and overlap with ND7 and CYb genes

The 5′ portion of the edited *T.brucei* COIII mRNA sequence was determined by a combination of RNA sequencing and sequencing of PCR products obtained using an edited COIII primer and a primer corresponding to the expected 5′ unedited region of COIII. We were unable to obtain clean RNA sequence for the 5′-most nucleotides but observed a strong primer extension product with sufficient laddering in the sequencing lanes to predict the 5′ end of the transcript (data not shown). To verify the terminal sequence and location of the 5′ end, clones derived from anchor PCR analysis were sequenced. Clones which ended at the predicted 5′ end were obtained from both BF (three clones) and PF (five clones) form RNA, although only one of the PF form clones matched the fully edited consensus sequence derived from analyses of prior conventional PCR clones.

The complete edited COIII mRNA sequence is presented in Fig. 1. The edited mRNA sequence of COIII from *T.brucei* EATRO 164 differs from that of strain EVE10 (23) only at three editing sites (Fig. 1). The EATRO 164 sequence contains three Us at editing site 13, versus two in EVE10; two at editing site 11 versus three in EVE 10; and none at editing site 352 versus one in EVE 10. In addition, EATRO 164 contains two additional G residues at its 5′ terminus. None of these sequence differences affect the protein coding capacity of the mRNA, since they occur outside the open reading frame.

The COIII gene overlaps both of the surrounding genes, CYb (22) and ND7 (6). The sequences for the 3′ end of the COIII gene and the 5′ end of the CYb gene overlap by one to five bp. The 5′ end of COIII mRNA is encoded in a sequence that also encodes at least 25 nts of the 3′ end of ND7 mRNA, however, the overlap could be as much as 33 nts. The uncertainty in length

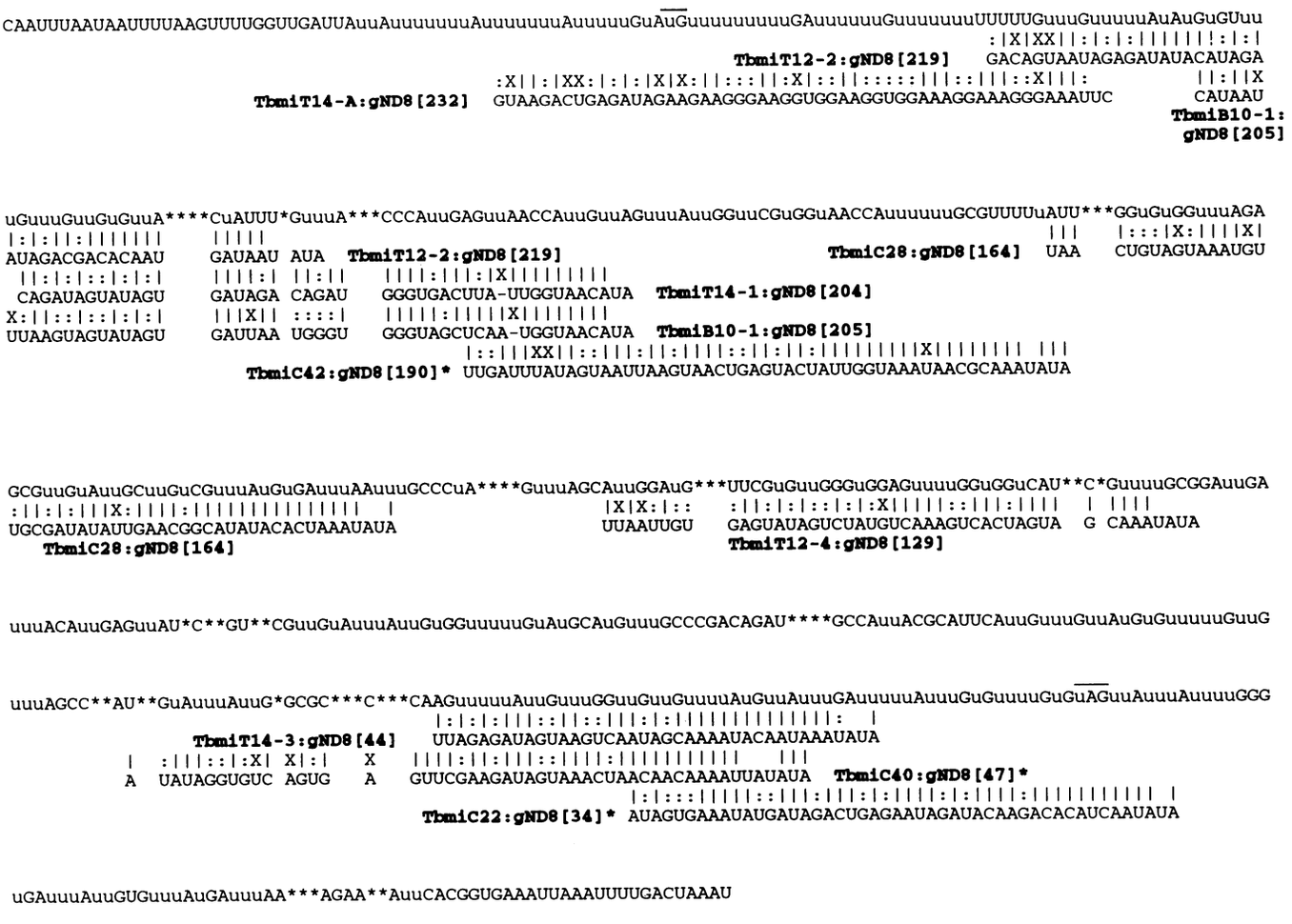

Figure 5. Alignment of gRNAs with edited ND8 mRNA sequence. Other symbols as in Fig. 1.

of overlap reflects AUs in the 3′ end of the cloned cDNAs that could either be encoded or added by editing or polyadenylation. The 3′ end of ND7 mRNA is edited at one site that is encoded in the overlap and the 5′ end of COIII mRNA is edited in as many as three sites. Thus, while it is conceivable that a single polycistronic precursor molecule could give rise to COIII and CYb mRNAs, this cannot be the case for a ND7/COIII precursor unless a mechanism for sequence addition to mRNA exists.

gRNAs in *T.brucei* direct editing of substantially overlapping sequence

Computer analysis of 13 published minicircle sequences (14,21,24−28) and 11 unpublished complete minicircle sequences (see Materials and Methods), and 17 minicircle cassette sequences (20), all from *T.brucei* except for two minicircles from *T. equiperdum*, enabled us to identify 17 potential gRNA genes for COIII (Fig. 1), 12 for ND7 (Fig. 3), 11 for A6 (Fig. 4), and 10 for ND8 (Fig. 5). Of these 50 gRNAs, 32 specify editing of a region of mRNA which overlaps with that specified by another gRNA (Table I). The overlaps approach a continuum from as few as 2 nt (gND8[164] and gND8[190]) to as many as 52 nt (gND7[177A], gND7[177B] and gND7[177C]). This differs from the situation in *L.tarentolae*, where the range of overlap is only 8−18 nt and usually is in the range of 10−14 nts. which approximates the length of the predicted anchor duplex (15). The overlap in *T.brucei* is often substantially more than that needed for the anchor duplex with the next gRNA. Thus, the options for gRNA utilization in *T.brucei* appear more complex than in *L.tarentolae*. In some cases, the gRNAs may be regarded as analogs since they edit essentially the same region of mRNA. These analogous gRNAs may have identical or closely related sequences (e.g., gND7[177A] and gND7[177B]) or their sequences may be significantly different (e.g., gCOIII[220] and gCOIII[223]). The G·U base pairing allows different cognate gRNAs to specify the same edited mRNA sequence.

The examples presented here represent the most gRNAs identified for any *T.brucei* mRNAs. However, not all of the gRNAs presented are from the same strain of *T.brucei*, nor have we tested for the existence of RNA transcripts for all of the gRNA genes identified. Inclusion of gRNAs from *T. equiperdum* is valid, since *T. equiperdum* appears to be a mutant form of *T.brucei* (Shu *et al.* submitted). While we have not verified the existence of all gRNA genes as RNA transcripts, a substantial proportion of the gRNAs presented (15 of 50) have been shown to exist as RNA (11,12,20,26,29,30). Every gRNA that has been investigated has been shown to exist, with one exception (12). In the majority of the gRNAs which have been analyzed by primer extension or cDNA cloning, the 5′ end of the gRNA

Table I

Gene	gRNA Type				
	Overlapping gRNAs[a]			gRNAs with mismatches[b]	
	gRNA	gRNA	gRNA	gRNA	gRNA
COIII	220–41	110–19	240–8	311–2*	147–2*
	223	102	226	220–2*	128–2*
				194–1	110–1
	168A–38	177–15	128–4	177–1	102–2
	168B	168	110	168–2*	62–2
	226–27	194–9	147–3		
	220	177	128		
	226–29				
	223				
ND7	177A–52	59–36	398–18	563–3*	177A–2*
	177B	65	385	550–2	150–1*
	177C			506–1*	59–2
				398–1*	65–5
				385–1*	
A6	138A–46	48–45	97–26	258–1	97–2*
	138B	51	92	149–3	92–2*
				138A–2*	48–4
				138B–5	51–4
				111–4	
ND8	204–44	44–19	47–10	204 –1	232–7
	205	34	34	205–4	219–3*
				190–4	129–3
	44–27	205		164–3	47–3
	47	204–13			
		190			

The gRNAs are identified by their editing site; full gRNA designations are in the figures.
[a]overlapping gRNAs are grouped and the amount of overlap is shown in nts.
[b]The numbers indicate mismatches between a gRNA their cognate gRNA region. The gRNAs with mismatches only in their last ten nucleotides are indicated with asterisks.

corresponds to the RYAYA box postulated to be the transcription initiation site(12). The 3′ end of most gRNAs appears somewhat variable, but is generally several nucleotides downstream of the sequence matching the mRNA (11,12)(Stuart et al., unpublished data). Although we have yet to identify all the gRNAs for any individual edited gene in *T.brucei*, the expected number of gRNAs for editing these transcripts can be estimated from the available data. ND7 has 1,128 nucleotides of final sequence created by editing, including those necessary to form the anchor duplex with the 3′ gRNA of each of the two editing domains. The 12 identified gRNAs (Fig. 3) are complementary with 337 nucleotides of fully edited sequence. By simple extrapolation, approximately 36 gRNAs would be expected to complete the editing for ND7. Similar calculations suggest 30 gRNAs for COIII, 21 gRNAs for A6 and 18 gRNAs for ND8, and extending these calculations to all of the edited *T.brucei* mRNAs predicts a total of at least 200 gRNAs. This is an underestimate since some gRNAs overlap by only 2–6 nt (e.g. gND8[164] and gND8[190], Table I), indicating a need for at least one additional individual gRNA. In addition, we cannot identify the cognate mRNA for about 30% of the gRNA genes we have sequenced. Nevertheless, *T.brucei* has ample coding capacity. It contains 300–400 different minicircles and thus can encode about 1200 different gRNAs. This implies an even greater gRNA overlap and redundancy than observed.

T.brucei gRNAs often contain mismatches with edited mRNA

Of the 50 gRNAs identified here, only 14 could form a perfect duplex between the cognate gRNA and fully edited mRNA, even when allowing G·U base pairing. The other 36 contain 1–7 mismatches (i.e. not A·U, G·C or G·U base pairs) or gaps (see Table I). Fourteen of these contain the mismatches solely within the 3′ 10 nt of the gRNA, but in the remaining 22, the mismatches are scattered throughout the gRNA as one or two mismatches in the midst of otherwise perfect alignment. The most frequent mismatch was U·U, although most (24 of 31 sites) of these occurred in the 3′ 10 nt of the gRNA. A·G and G·G mismatches were also common, but tended to be scattered throughout the gRNA (7 of 14 and 6 of 9 sites, respectively, not in the 3′ 10 nts). C·A, A·A and C·U (3 of 5, 1 of 6, 0 of 9 sites, respectively, not in the 3′ 10 nts) mismatches occurred less frequently throughout the gRNA.

DISCUSSION

We present here the complete edited COIII mRNA sequence of *T.brucei* and the overlaps of its gene sequence with those of the flanking ND7 and CYb genes. The elucidation of the edited sequence for COIII, ND7, ND8, and A6 mRNA allows us to identify 46 gRNA genes that can specify the editing of these

mRNAs. The COIII edited mRNA differs at five positions from that of another strain (EVE 10). However, all of these differences, two of which are at the 5′ terminus, lie outside the open reading frame of the message and possibly reflect gRNA gene divergence. This resembles, in a less dramatic fashion, the differences between the fully edited COIII mRNAs of *T.brucei* and *T.congolense*. These edited mRNAs differ more in the 5′ and 3′ untranslated regions than in the coding region, and differences in the latter do not change the predicted amino acid sequence (Read *et al.* submitted for publication). These results are consistent with evolutionary conservation of the expressed protein sequence. There is considerable overlap between the COIII gene and those of the flanking ND7 and CYb genes, respectively, as previously seen for CR6 and ND5 (31). Since all three mRNAs are transcribed from the same strand and are edited in the region of overlap, it is clear that ND7 and COIII mRNAs cannot be derived from the same precursor molecule.

The gRNAs presented specify editing of substantially overlapping sequences in the COIII, ND7, A6, and ND8 mRNAs in *T.brucei*. There is a continuum in the sizes of the overlaps that range from very short where the 3′ gRNA does not specify the sequence for an anchor duplex with the 5′ gRNA to overlaps where analogous gRNAs direct editing of essentially the same region of their cognate mRNAs. This situation differs substantially from *L.tarentolae* where it has been reported that the gRNAs only overlap enough for one gRNA to create the sequence necessary for an anchor duplex with the next gRNA (26). In addition, there appears to be a single *L.tarentolae* gRNA sequence for each section of edited mRNA. This greater gRNA complexity in *T.brucei* than in *L.tarentolae* parallels the greater minicircle complexity. It implies low degrees of freedom in the order of gRNA utilization in *L.tarentolae* but more diversity of their order of utilization in *T.brucei*.

The extensive overlap of the gRNAs in *T.brucei* may provide a form of proofreading not present in *L.tarentolae* since incomplete editing by one gRNA to an sequence may be corrected by another gRNA. This may be important in *T.brucei* where total editing is more extensive than in *L.tarentolae* and *Crithidia fasciculata*. It may also in part explain the tolerance of gRNA/mRNA mismatches. Thermodynamic considerations may necessitate such a multi-step process in *T.brucei*. The greater total complexity of the gRNA population in *T.brucei* may lead to more gRNAs being used at non-cognate locations, as has been seen in *L.tarentolae* (32). This non-cognate editing may be corrected by the editing with the cognate gRNA, which could be more efficient if several analogous gRNAs were present.

The instances of extreme overlap between gRNAs in *T.brucei* suggest that there is a redundancy in the gRNAs present in this organism. While two pairs of minicircle sequences were derived from different strains (gCOIII[220] gCOIII[223], and COIII[168A] gCOIII[168B]) and it is not known whether both analogous gRNAs exist in EATRO 164 there are several instances where analogous gRNAs were derived from the same strain. Some of these analogous gRNAs are clearly derived from closely related minicircles (e.g. gND7[177A], gND7[177B] and gND7[177C] have very similar or identical sequences). However, in other cases (e.g. gA6[48] and gA6[51]) the sequences are much less related. Cognate gRNA redundancy has clear evolutionary implications. It may allow for mutation and/or loss of one gRNA gene without the loss of protein coding capacity because the analogous cognate gRNAs would still direct editing of the mRNA. This may help explain why several genes (i. e., ND8, ND9, CR3, CR4, and CR5) are edited in *T.brucei* but not in *L.tarentolae* (15,30). Some gRNA genes for editing these mRNAs appear to have been lost in *L.tarentolae* (15). Conversely, the presence of multiple analogous gRNAs may allow mutation of some gRNAs and create different edited mRNA sequences. The resultant mutant proteins may be expressed along with the normal proteins. This may provide for selection of beneficial mutations, without the selective disadvantages of detrimental mutations. Thus, the presence of redundant analogous gRNAs in *T.brucei* may allow the accelerated evolution of mitochondrial gene products in this organism.

The majority of the gRNAs we have identified contain some mismatch with their cognate edited mRNA. Most of these mismatches occur in the last 10 nt of the gRNA-mRNA duplex and may have little significance since the extensive gRNA overlapping in *T.brucei* suggests that these sites are likely to be re-edited. Internal mismatches have been seen in other gRNA-mRNA duplexes (30,33). These may be tolerated by the mechanism of RNA editing as previously suggested (29) or compensated for by gRNA redundancy. The model presented by Koslowsky and colleagues (29) predicts that the choice of editing site is driven by thermodynamic interactions and editing continues until the most thermodynamically stable interaction between the gRNA and the mRNA is achieved; this final interaction could include mismatches. Some of the mismatched gRNAs may represent gRNA gene divergence as appears to be the case for two A6 gRNAs encoded in related minicircles that also encode CYb gRNA and CR3 gRNAs (Fig. 4, and Riley *et al.*, submitted for publication). One A6 gRNA has no mismatches with the mRNA, while the other has five mismatches to the same mRNA region. It is of interest to note that CR3 and CYb gRNAs encoded on both minicircles contained fewer or no mismatches.

Our results indicate a much greater complexity of the gRNA population and its interaction with mRNA during editing in *T.brucei* than described for *L.tarentolae*. This may reflect more complex editing control mechanisms in *T.brucei*, where editing is differentially regulated between life cycle stage in a transcript specific manner (6,22,30,34,35). It may also represent an adaptation that increases the rate of evolution of edited gene products.

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