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Mini review

The genomic organization of guide RNA genes in kinetoplastid protozoa: several conundrums and their solutions

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Abstract

The guide RNA (gRNA) paradigm states that the uridine (U) insertion/deletion type of RNA editing is mediated by short 3′ uridylylated gRNAs that are complementary to specific blocks of mature edited sequence. These gRNAs contain the edited sequence information in the form of guiding purine residues that can base pair with the inserted U's and do not base pair with encoded U's that are to be deleted. The minicircle gRNA genes in trypanosomatids are localized at specific sites within the variable region, with the number and the precise localization of genes also being species-specific. The total number of minicircle sequence classes and thereby minicircle-encoded gRNAs varies greatly between species and even between different strains of the same species, with the greatest number being in the trypanosome species. Several conundrums which appeared to raise problems for the gRNA paradigm arose during comparative analysis of minicircle gRNA gene organization. The solution of these conundrums has led to a better understanding of the function and evolution of this RNA modification phenomenon. © 1997 Elsevier Science B.V.

Keywords: Kinetoplastid protozoa; RNA; Conundrums

1. Introduction

The kinetoplastid protozoa consist of at least two major groups, the trypanosomatids and the bodonids/cryptobiids. The mitochondrial DNA of the trypanosomatids, which exists as a large nucleoid body composed of a single giant network of DNA, is unusual in that it consists of two separate genomes the RNA products of which interact to produce mRNAs which are presumably functional for translation [1–3]. The maxicircle, which is present in approximately 20–50 copies per network, contains two rRNA genes, structural genes, and cryptogenes, the open reading frames of

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which are defective to varying extents. The minicircle component is highly polyploid (10–20 000 copies per network), and contains guide RNA (gRNA) genes, transcripts of which mediate editing of the cryptogene mRNA transcripts [4,5]. The minicircle is organized into one to four conserved regions representing approximately 10% of the molecule and an equal number of variable regions, depending on the species. The gRNAs are short transcripts that have a 3′ non-encoded oligo[U] tail approximately 13 U's in length. The gRNAs also have a di- or tri-phosphate at the 5′ end that can be labeled with [α-^{32}P]GTP by guanylyltransferase [6], raising the possibility that they represent primary transcripts. The 5′ region of each gRNA can base pair with mRNA sequence just downstream of a specific pre-edited block to form an anchor duplex, and the entire gRNA except for the oligo[U] tail forms a complete duplex with the fully edited mRNA. Several models for the mechanism of this type of editing have been proposed. Recent in vitro evidence provides support for the original enzyme cascade model [7–12].

The cryptobiid kinetoplastid, *Trypanoplasma borreli*, also contains a single large nucleoid body within the single mitochondrion. Little was known until recently about the molecular biology of the mitochondrial genome in these cells.

2. Five conundrums

2.1. Loss of minicircles and gRNAs of L. tarentolae in culture

When the total minicircle-encoded gRNA repertoire in the old UC laboratory strain was determined by an exhaustive search of a minicircle library, it was found to be insufficient for mediating the editing of the transcripts of all the known cryptogenes [13]. This represented a problem for the gRNA paradigm.

The initial seven gRNAs identified in 1990 were found to be encoded in the maxicircle DNA [14]. An additional eight maxicircle-encoded *L. tarentolae* gRNAs were identified, as new edited mRNA sequences became available [15]. The gRNAs have unique 5′ ends which could be capped with [α-^{32}P]GTP and guanylyltransferase [6]. The discovery of two gRNA genes in *L. tarentolae* minicircles provided the first explanation for the genetic role of these enigmatic molecules [4]. One gRNA could mediate editing of the first eight sites in the COIII mRNA and the other could mediate the misediting of a partially edited COIII mRNA sequence. The minicircle in *L. tarentolae* is organized into an approximately 170 nt conserved region, that contains the origins of replication for both strands, and a variable region that defines the specific minicircle sequence class (Fig. 1). The 12 nt CSB-3 sequence (GGGGTTGGTGTA), which is conserved in minicircles from all trypanosomatids [16], provides a relative position and polarity marker for gRNA genes. A region of bent DNA, apparently due to phased runs of A's, is situated immediately adjacent to the conserved region. All gRNA genes so far identified are localized within the variable region approximately 300 bp from the CSB-3 sequence and 150 bp from the bend region (Fig. 1).

Additional minicircle-encoded gRNAs from the *L. tarentolae* UC strain were identified by constructing libraries from permuted MspI-linearized minicircles and performing an exhaustive negative colony hybridization search using oligonucleotide probes to known gRNAs [13]. By this method an additional 12 minicircle sequence classes were discovered. Inspection of the minicircle sequences at the known gRNA loci within the variable regions and comparison to known edited mRNA sequences identified the encoded gRNAs. The existence of these gRNAs was further corroborated by Northern analysis and direct sequencing of the 5′ ends, and by identification in a gRNA library [15]. This gRNA repertoire appeared to be exhaustive for the UC strain of *L. tarentolae*, but it was clearly insufficient to mediate the editing of all the pre-edited mRNA products of the G1–G5 cryptogenes [15].

The UC strain was isolated in Algeria in 1939 and has been maintained in culture in various laboratories since then. We decided to examine the gRNA repertoire in a strain of *L. tarentolae*, LEM125, which was more recently isolated from a natural host. A gRNA library was constructed

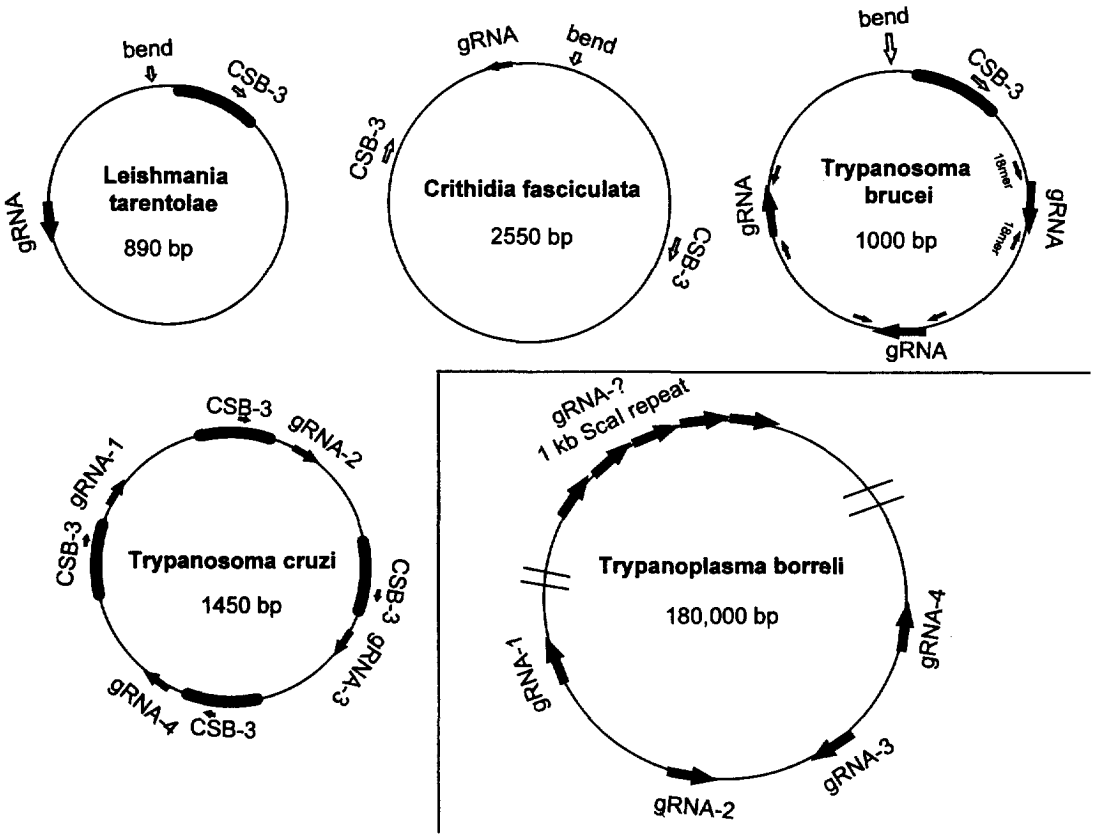

Fig. 1. Comparison of gRNA gene organization in minicircle DNA from several trypanosomatid species and in the 180 kb DNA from *T. borreli*. The size of the minicircle for each species is indicated as well as the location and polarity of the conserved region containing the CSB-3 sequence [16], the bend (if present), and the gRNA genes. It is not established if the identified gRNA genes in the *T. borreli* DNA are in the same molecule as the ScaI gRNA genes as shown, nor is it known if the polarity of the gRNA genes is as diagrammed.

from this strain and an additional 32 minicircle-encoded gRNAs were identified which were present in this strain but were absent in the UC strain [15]. All of these gRNAs were specific for the editing of products of transcripts of five G-rich regions (G1–G5), which were presumed to be pan-edited cryptogenes. Several of the transcripts of G1–G5 may encode components of complex I of the respiratory chain. It was estimated that an additional 23 gRNAs which were required for the complete editing of these transcripts still remained to be identified. These findings led to the hypothesis that specific minicircle sequence classes encoding gRNAs for the editing of the G1–G5 transcripts had been lost during the long culture history of the UC strain. This loss is perhaps due to missegregation of the minicircles at mitochondrial division in the absence of a selective pressure in culture for the protein products of the edited transcripts. This process of random loss of minicircle sequence classes could also be the driving force for the replacement of pan-edited cryptogenes with cDNA copies of partially edited transcripts, giving rise to 5′-edited and even unedited genes, a process which appears to have occurred during evolution of the trypanosomatids [17,18].

Homologues of the two unassigned gRNAs in the UC strain, gLt19 and gB4, were shown to encode editing information for block III of G4 and block IX of G5 (= ND3) in the LEM125

strain [15]. Due to the absence of additional gRNAs in these editing cascades in the UC strain, the gLt19 and gB4 gRNAs are not involved in a productive editing cascade. The G4 and ND3 maxicircle sequences in the UC strain, as well as G1–G3, therefore represent 'pseudocryptogenes', which are defined as cryptogenes whose transcripts are not productively edited. Interestingly, the two minicircle sequence classes encoding the G4-III and ND3-IX gRNAs represent the most abundant classes in the UC strain, and the copy number of these (and other minicircles) was found to fluctuate dramatically during a 2-year culture period of the UC strain, as well as between the UC and LEM125 strains (O. Thiemann and L. Simpson, unpublished results). However, no correlation was observed between minicircle copy number and gRNA relative abundance, indicating the lack of a gene dosage effect. We have speculated that the Lt19 and B4 minicircles in the UC strain were used as a buffer to maintain the total number of minicircles required for network integrity [15].

In *L. tarentolae* the identified gRNAs generally form sets of minimally overlapping sequences, in which the anchor for the upstream gRNA is created by editing of the adjacent downstream sequence [13]. Only a few examples of extensively overlapping or 'redundant' gRNAs of different sequences with the same editing information have been found [15]. The minicircle genome in this species seems to encode a minimal gRNA repertoire.

By sequencing a collection of clones from a gRNA library obtained by 3′/5′ RACE (rapid amplification of cDNA ends), the 3′ uridylylation sites for each specific gRNA were found to be remarkably homogenous [19]. It is not known if primary transcripts terminate at the observed 3′ ends or if there is endonucleolytic cleavage prior to terminal addition of uridylylate residues by the mitochondrial terminal uridylyl transferase.

2.2. The homogeneous minicircle DNA of C. fasciculata

The kDNA network in the C-1 strain was reported to consist of approximately 5–10 000 minicircles 2500 bp in size and homogeneous in sequence [20,21]. However, sequences of several pre-edited cryptogenes and mature edited transcripts indicated that the extent of editing in this species was essentially identical to that in *L. tarentolae*. These findings raised the question of the capacity of the minicircle DNA to encode the required variety of gRNAs.

The minicircle in *C. fasciculata* is organized into two antipodal conserved regions and two variable regions [21,22] (Fig. 1). A single bend region is situated 90° from the conserved region within one of the two variable regions.

A gRNA library was constructed from total mitochondrial RNA and the sequences of randomly selected clones were compared with the fully edited mRNA sequences for two pan-edited genes, RPS12 and A6. Five gRNAs were identified, two of which edit blocks within the A6 mRNA and three of which edit blocks within the RPS12 mRNA [23]. Little heterogeneity was observed in the 3′ uridylylation sites of the gRNAs; this differed from the extensive 3′ heterogeneity reported for maxicircle-encoded gRNAs in the Steinert strain of *C. fasciculata*, which was invoked as a mechanism that would give rise to an appearance of 3′ to 5′ polarity of editing within an editing block [24]. Clearly, this explanation is not consistent with the 3′ end results obtained with the C-1 strain of *C. fasciculata* and with *L. tarentolae*.

The genes for all five of these gRNAs were localized within one of the two variable regions of specific minor minicircle molecules, approximately 60 bp from the bend region. The specific localization of all five genes suggests that this represents the general location of gRNA genes in this molecule. All five minicircles represented minor sequence classes comprising approximately 2% of the minicircle population in the network. An unassigned gRNA gene was also identified at the same relative location in the major minicircle sequence class which represents over 90% of the kDNA. The minicircle DNA and gRNA complexity of the Steinert strain [25], which was shown to represent a different 'schizodeme' [26] than the C-1 strain [23], was not analyzed. These results suggest that the 'single minicircle' paradox is due

to an amplification of one specific minicircle sequence class with no overall effect on the RNA editing system due to the retention of minor minicircle sequence classes which encode the required variety of gRNAs. This is also consistent with a lack of correlation of gRNA relative abundance and minicircle copy number, as was found for *L. tarentolae*.

2.3. The excessive minicircle sequence complexity in T. brucei

Early studies of DNA renaturation kinetics [27] indicated a complexity of over 200–300 minicircle equivalents for the total kDNA from *T. brucei*. Since more than 90% of the kDNA consists of 1 kb catenated minicircles, this indicated the presence of several hundred different minicircle sequence classes. Furthermore, the presence of three gRNA genes in each minicircle [5] suggested that there were over 600–900 different gRNAs. These results raised the question of the role of the large number of different gRNAs in this species, which appeared to be much greater than required for the editing of the known pre-edited mRNAs.

The minicircle molecule in *T. brucei* and the closely related *Trypanosoma equiperdum* and *Trypanosoma congolense* is organized into a single conserved region of approximately 120 bp and a single variable region [28] (Fig. 1). A DNA bend is located at the edge of the conserved region [29]. The variable region encodes three gRNA genes which are situated between three sets of imperfect 18-mer inverted repeats [30] (5′-GAAATAAGATAATAGATA-3′/5′- TATTTATTATTTTATTTT-3′). Three examples of redundant 'orphon' gRNA genes, which are not located between inverted repeats, have been observed, all of which can mediate the editing of block I of the cytochrome *b* mRNA [31]. All of the orphon Cyb gRNA genes are located within the same minicircle sequence class, and two of the gRNAs have 20 nt 3′ extensions, possibly suggesting a defect in termination of transcription or in 3′ end processing.

Analysis of random minicircle sequences from *T. brucei* and *T. equiperdum* has led to the identification to date of over 80 different gRNAs. It should be noted that these gRNAs are derived from different strains of *T. brucei*, and the contribution of strain heterogeneity to gRNA sequence heterogeneity is not yet determined for this species. A complete set of overlapping gRNAs has not been identified for any editing cascade in *T. brucei* and it is not certain if there really will prove to be over 900 different gRNAs. It is clear, however, that the repertoire of minicircle-encoded gRNAs is much larger than in *L. tarentolae* and *C. fasciculata*. In addition, the gRNA sequences overlap greatly in a continuum from as little as 2 nt to as many as 52 nt, suggesting that the anchor sequences are not restricted to unique locations. The existence of such extensive redundant gRNAs is the most likely explanation of the excessive minicircle sequence complexity in *T. brucei*. However, an explanation for the existence and retention of this large gRNA repertoire must be found in the evolution of the editing system in the trypanosomatids.

2.4. The heterogeneous minicircle DNA of Trypanosoma cruzi

The kDNA in *T. cruzi* was known for many years to be extremely heterogeneous in sequence and to vary in different strains and isolates. The complex restriction enzyme digestion patterns observed in acrylamide gels were in fact used for distinguishing strains or schizodemes of this parasite [26].

Analysis of the sequences of several 1.5 kb minicircles showed the presence of four conserved regions approximately 120 bp in size and four variable regions situated 90° apart [32] (Fig. 1). Alignment of the conserved regions from multiple trypanosomatid species showed the presence of three short highly conserved sequences. These sequences were used to devise primers for polymerase chain reaction (PCR) amplification of the adjacent variable regions (VR) as a diagnostic tool [33]. Digestion of the amplified VR DNA with restriction enzymes provided additional evidence for an extremely high degree of interstrain sequence heterogeneity in *T. cruzi* [34]. No evidence for the presence of a bent DNA region, such as exists in all other trypanosomatid minicir-

cles, was obtained by comparison of electrophoretic mobility on agarose and acrylamide gels [32].

To identify specific gRNAs, gRNA libraries were constructed for two clonal strains, Sylvio and Can. Sequences from random clones were compared with the MURF4 edited mRNA sequence [35]. By this method, 18 MURF4-specific gRNAs were identified, covering approximately 90% of the edited sequence. A minicircle VR library was then constructed and the sequences of random clones tested for gRNA-like base pairing potential with the MURF4 edited mRNA sequence. Nine Sylvio VR clones contained putative MURF4 gRNAs and three of these matched gRNAs from the gRNA library. All of the identified gRNAs were localized within the variable regions 60–100 bp from the CSB-3 sequence (Fig. 1). An additional 30 gRNA clones were found to be encoded in minicircle VR sequences at similar locations.

At least five pairs of redundant gRNAs were present in the identified and unassigned gRNAs from the same strain [35]. In addition several pairs of 'homologous' gRNAs (i.e. redundant gRNAs derived from different strains) were also identified. The sequence differences between redundant/homologous gRNAs mainly involve transitions, which in most cases do not interfere with the gRNA/mRNA complementarity, but are sufficient to affect hybridization. The alignment of VR sequences from Sylvio and Can that encoded redundant and homologous gRNAs showed that the VR sequences were derived from common ancestral sequences, but accumulated random polymorphisms both within the gRNA and in the upstream and downstream VR regions; this is consistent with the hypothesis that *T. cruzi* strains in nature are clonal and have been genetically isolated for long periods of time [36]. These results solve the challenge to the gRNA paradigm presented by the extensive minicircle sequence heterogeneity between different strains of *T. cruzi*.

2.5. The absence of minicircles in Trypanoplasma borreli

T. borreli is the only representative of the bodonid/cryptobiid lineage which can be cultured axenically and is therefore amenable to molecular analysis. In spite of the existence of a cytological kinetoplast-like structure in the mitochondrion, no evidence for the presence of a network of catenated minicircles was obtained. The kDNA was found to be composed of two classes of circles, 40–80 kb and 200 kb in size [37]. Two overlapping fragments of the 40–80 kb molecules were sequenced and found to encode several recognizable mitochondrial genes in addition to several G-rich sequences [37,38]. Unedited COII and COIII genes, and 3′ and 5′-edited Cyb and COI genes were identified. In addition, one of the G-rich sequences was shown by analysis of partially edited transcripts to encode a pan-edited RPS12 cryptogene, and editing appeared to proceed 3′ to 5′ as in the trypanosomatids. However, the gene order and polarity is different from that found in the trypanosomatid maxicircle genome, which is consistent with the ancient divergence of these lineages [17]. The conundrum is that these cells have uridine insertion/deletion editing but lack minicircles, which in the trypanosomatids encode the majority of gRNAs.

The presence of gRNA-like molecules in this species was indicated by capping total cell RNA with [α-^{32}P]GTP and guanylyl transferase and observing a smear of labeled small RNAs, 40–60 nt in size, migrating ahead of tRNA in acrylamide [37]. This RNA hybridized to a 1 kb tandemly repeated fragment released from the 180 kb circles by digestion with ScaI. Construction of a library of small RNAs by 3′/5′ RACE led to the identification of five gRNAs encoded by sequences not present in the ScaI repeats which could mediate the editing of short blocks of the RPS12, COI and Cyb mRNA sequences [39]. All of these gRNAs had short oligo[U] sequences at the 5′ end, in addition to oligo[U] at the 3′ end as in gRNAs from trypanosomatids. The genes for two of these gRNAs were cloned and found to be located within the 180 kb circle component (Fig. 1). Neither the 3′ nor the 5′ oligo[U] sequences were encoded in the DNA. The mechanism for the addition of the 5′ non-encoded homopolymer sequence is unknown, as is the possible genetic function of such a sequence.

An unassigned gRNA was also identified which was derived from the 1 kb ScaI repeat sequence. This repeat sequence was shown to be approximately 1000 times more abundant than the identified gRNA genes, indicating a heterogeneity of the 180 kb molecules.

The presence of a single putative gRNA-encoding sequence at 1000 times the frequency of the individual identified gRNA genes is reminiscent of the situation in the C-1 strain of *C. fasciculata*, in which more than 90% of the kDNA is a single minicircle sequence class [23]. This again illustrates the plasticity of the gRNA gene component of the kDNA, in which individual genes are found to vary considerably in relative frequency without apparent effect on the editing system. The presence of gRNA genes in tandem in a 180 kb circular molecule is of great interest, and it is intriguing to speculate that this represents the evolutionary precursor of the minicircular type of gRNA gene organization in the trypanosomatids. One could envisage each gRNA gene unit acquiring an autonomous replicating sequence and becoming a self-replicating circular plasmid. Catenation of the circles into a network would greatly limit the loss of individual molecules at mitochondrial and cell division and perhaps even serve another role, such as in enabling the proper segregation of the maxicircle molecules which contain the structural genes and cryptogenes. Comparative investigations of the phylogenetic status and gRNA gene organization of additional bodonids and cryptobiids will be necessary to illuminate this situation.

3. Conclusions

Solutions of these apparent conundrums which at first appeared to be inconsistent with the gRNA paradigm have in fact strengthened the paradigm and led to several important conclusions. The major conclusion concerns the plasticity of the gRNA gene repertoire, both during prolonged growth in culture and during evolution. The frequency of individual gRNA genes can vary dramatically with little apparent effect on the editing system until a complete loss of a specific gene class occurs. Another conclusion is that several of the trypanosomatid species, such as *T. brucei* and *T. cruzi*, would probably not even be affected by the complete loss of a specific gRNA class, since there are redundant gRNAs which encode the same editing information. It is striking that, in spite of the plasticity of minicircle sequence class frequencies, the total number of catenated minicircles in the network is maintained, suggesting the presence of an alternate and perhaps a structural role for minicircle DNA and the minicircle network. The evolutionary origin of the gRNA genetic system in the kinetoplastids is still an unsolved problem. It is however clear that the minicircular type of gene organization is not essential since editing occurs in *T. borreli* which has a non-minicircular type of gRNA gene organization. Further comparative molecular investigations should greatly increase our knowledge of these important and interesting problems.

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