# Ancient origin of RNA editing in kinetoplastid protozoa

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A rooted phylogenetic tree of the kinetoplastid protozoa has been constructed that, together with a comparative analysis of editing of several genes, leads to the surprising conclusion that extensive or pan-editing with multiple overlapping guide RNAs is more ancient than 5′-editing. The mechanism of editing is still uncertain, but multiple ribonucleoprotein complexes have been identified that contain components of the enzymatic machinery.

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## Introduction

The U-insertion/deletion editing of transcripts of the mitochondrial maxicircle DNA in trypanosomatid protozoa remains one of the most unusual molecular phenomena yet described. Discovered in 1986 [1] as the post-transcriptional insertion of a few U residues in the cytochrome oxidase (CO) subunit II (COII) mRNA in *Crithidia*, editing was soon found to also include deletions [2] and to occur at multiple sites at the 5′ end of 'editing domains' (5′-editing) [2] or throughout the coding (and 3′ non-coding) sequences of several mRNAs (pan-editing) [3–6,7•–9•]. The edited sequence information was found to reside in small 3′ oligo-uridylated guide RNA (gRNA) molecules that could form anchor hybrids with pre-edited RNAs and mediate the insertion and deletion of Us at precise sites by a mechanism that involves base-pairing [10,11]. Some gRNA genes have been found in maxicircle DNA, but the majority have been localized in minicircle DNA molecules [12–15,16••], which are linked to maxicircles and other minicircles by catenation to form a giant network of DNA in the single mitochondrion [17].

Two basic models for editing have been proposed. The first [11,18] involves cleavage, addition of Us either directly from UTP or from the 3′ oligo(U) tail of the gRNA, and ligation. The second [19,20] involves two successive transesterifications resulting in the transfer of Us from the 3′ end of the gRNA into the editing site. The observed 3′ to 5′ polarity of editing within a domain [21,22] has been shown to be the consequence of the formation of upstream anchor sequences by editing of the downstream mRNA sequence [16••,23,24]. The appearance of unexpected editing patterns in partially edited RNAs at junction regions, however, has led to the proposal that editing can occur at random sites and that the correct edited sequence is frozen by base-pairing with the gRNA [25]. An alternative proposal is that unexpected editing patterns are actually examples of 'misediting' as a result of 'misguiding' [26]. Several extensive reviews of this field have been published recently [27–32]. In this review, we summarize the results of recent studies that have not only provided a better understanding of the mechanism and genetics of RNA editing, but have also overturned completely the established paradigm of its evolution.

## Phylogeny of kinetoplastid protozoa

Previous phylogenetic reconstructions using 18S rRNA sequences led to the conclusion that the kinetoplastid protozoa and the euglenoid flagellates represent the earliest divergence of mitochondria-containing eukaryotic cells [33]. In the past year, several laboratories have derived similar phylogenetic trees of the kinetoplastids, rooted using *Euglena* 18S rRNA as an outgroup [34••–36••] (Fig. 1). The existence of two major sister groups, the bodonid/cryptobiids and the trypanosomatids [37], has been confirmed by the molecular data. In addition, a surprising result has been obtained that within the trypanosomatids, the digenetic (two hosts) African trypanosomes (*Trypanosoma brucei*) and South American trypanosomes (*Trypanosoma cruzi*) represent the most deeply diverged groups, whereas the monogenetic (one host) *Crithidia* and *Leptomonas* and the digenetic *Leishmania* and *Endotrypanum* together form a monophyletic clade that diverged relatively recently. Several monogenetic species have been determined to have diverged prior to this clade. These results suggest either several independent losses of the digenetic life cycle [35••] or several independent gains [34••]. The divergence of the trypanosomatid lineage itself has been estimated to have occurred at approximately the time of the divergence of the vertebrates [34••].

**Abbreviations**

**CO**—cytochrome oxidase; **gRNA**—guide RNA; **ND**—NADH dehydrogenase;
**RNP**—ribonucleoprotein; **TUTase**—terminal uridylyl transferase.

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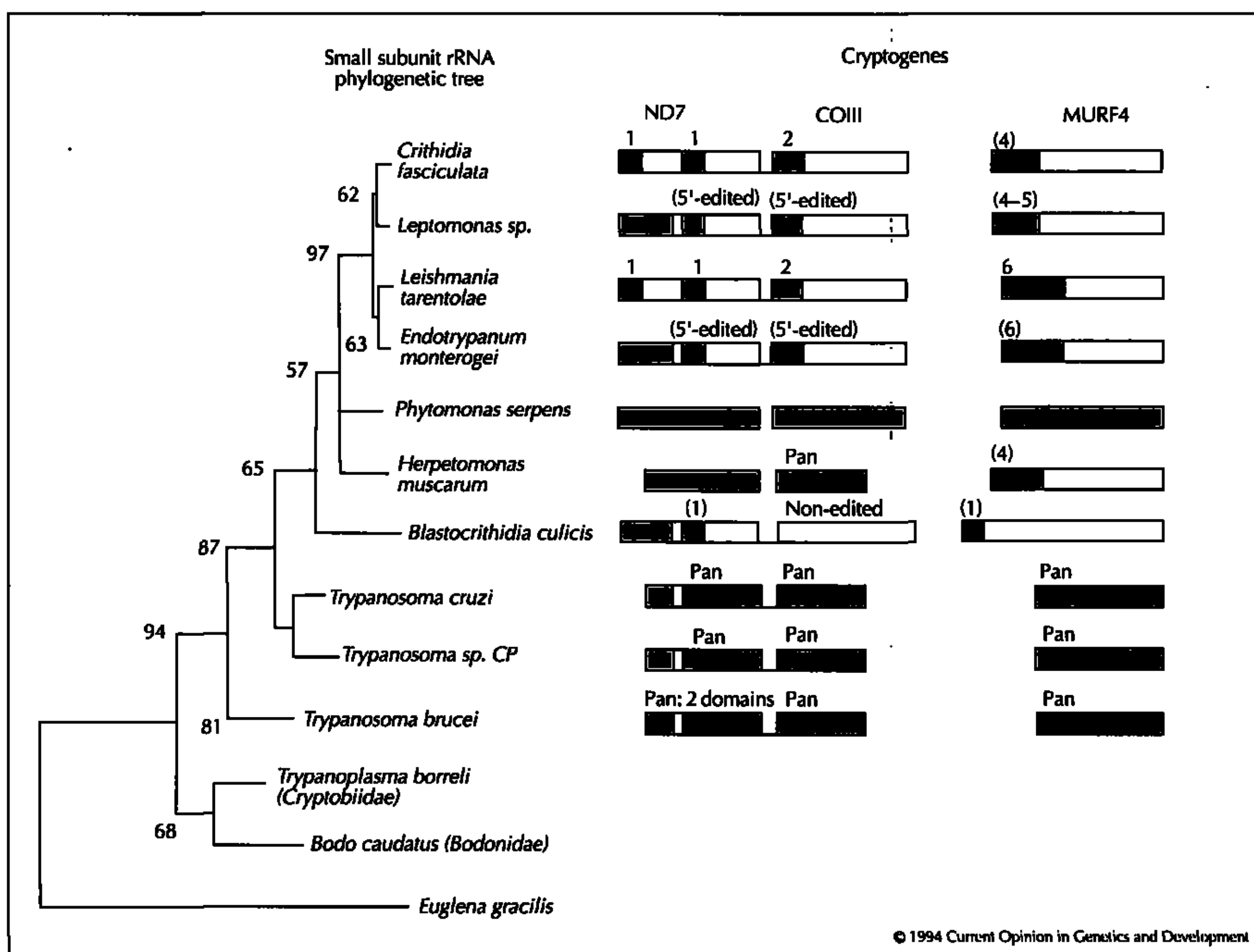

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Fig. 1. Phylogeny of kinetoplastid RNA editing. Aligned 18S rRNA sequences were used to contruct a consensus phylogenetic tree using maxium parsimony. Bootstrap percentage values are indicated for each node. Other algorithms, such as maximum likelihood and genetic distances, gave similar topologies. The tree was rooted using the *Euglena gracilis* sequence as an outgroup. A diagrammatic representation of the extent of editing in maxicircle unassigned reading frame 4 (MURF4), NADH dehydrogenase subunit 7 (ND7), and cytochrome oxidase subunit III (COIII) is shown on the right. Darkly tinted boxes indicate pre-edited regions, white boxes non-edited regions, and lightly tinted areas a lack of information. Numbers above the pre-edited regions indicate the actual number of gRNAs involved (i.e. the number of editing blocks) or the estimated number of gRNAs required (in brackets). The type of editing is indicated above each domain. (5′-edited) indicates that this editing pattern was deduced solely from the sizes of amplified fragments and not from DNA or edited RNA sequences. Modified from [36••].

## Pan-edited cryptogenes

In the past few years, several new pan-edited cryptogenes have been described. These were identified originally as G-rich intergenic regions, termed G1–G6 in *Leishmania tarentolae* and CR1–CR6 in *T. brucei*, which were conserved in location and polarity, but not in sequence, between these two species [38]. It was proposed that these represent pan-edited cryptogenes [39], and this hypothesis has been verified for G6/CR6 in both species [40,41], and for CR1, CR2, CR4, and CR5 in *T. brucei*. The edited G6 mRNA sequence has been identified as a highly diverged ribosomal protein S12 homolog [40]. Pan-editing of this transcript in *L. tarentolae* occurs in three domains, but in *T. brucei*, only a single editing domain is found [41]. The CR1 transcript in *T. brucei* is pan-edited to yield a sequence that contains two iron-sulfur cysteine motifs and is homologous to the NADH dehydrogenase (ND) subunit 8 (ND8) [6]. The pan-edited CR2 transcript in *T. brucei* has proved to be a homolog of ND subunit 9 (ND9) [7•], the pan-edited CR5 transcript is possibly a highly diverged homolog of ND subunit 3 (ND3) [8•]. The pan-edited transcript of CR4 is not homologous to any database sequence [9•].

## Microevolution of cryptogenes

The evolution of coding sequences is restricted by selection at the level of the primary, secondary, and tertiary structure of the translated proteins. In the case of cryptogene transcripts, an additional selection occurs because of the necessity for base-pairing with cognate gRNAs. Mutations in cryptogenes that affect this interaction may, therefore, be tolerated only if they are accompanied by

compensatory mutations in gRNA genes. As editing is responsible ultimately for the number of U residues in the mRNA, however, mutations in the number and position of genomic Ts that would be lethal in other systems appear to be well tolerated in kinetoplastid mitochondria.

The microevolution of pan-edited cryptogenes has been considered in a number of recent papers. Read and coworkers [42•,43,44•] have compared the primary structure of pan-edited ND subunits 7, 8, and 9 and CO subunit III in two related species of African trypanosomes, *Trypanosoma congolense* and *T. brucei*. As expected, the divergence between homologous cryptogenes is found to be mainly in the number of encoded Ts, but surprisingly, these Ts are distributed non-randomly and occur mainly at deletion sites. As the result of editing, the final patterns of Us in the edited mRNAs are the same. A few purine transitions have been found, however, that represent silent replacements. As shown by a single identified *T. congolense* gRNA that can base-pair with ND8 mRNA from both species, such transitions do not affect base-pairing, as they occur at sites at which a U is found in the gRNA. These data may illustrate the initial steps in cryptogene evolution. Because of conservation of the editing patterns in the gRNAs, these mRNA sequence changes are not reflected in changes in the protein sequences.

What if gRNAs have themselves evolved? Because of G–U pairing, not all changes in gRNAs will result in changes in the editing patterns. This is exemplified by the discovery of several minicircle-encoded gRNAs for apocytochrome *b* in *T. brucei* that direct the same editing pattern despite differences in their sequences [45••]. Such redundant gRNAs are quite common in *T. brucei* [16••], but are rare in *L. tarentolae* [23,46].

If mutations in gRNAs are not silent, mutated gRNAs would specify misediting events, but they may also provide a source of genetic variability. This has been described for the pan-edited COIII cryptogenes from several species of *Herpetomonas* [47•]. As in the African trypanosomes, the genomic sequences differ mainly by the numbers of Ts and by a few purine transitions. The protein sequences, however, are substantially diverged. Comparison of the fully edited mRNAs has shown that 'editing frameshifts' are often observed when an increased number of inserted Us at one site is accompanied by a reduced number of Us at a nearby site. Such frameshifts account for half of the amino acid replacements. The most likely mechanism for this mode of cryptogene evolution involves mutations in gRNAs. As the result of these changes in the edited mRNA sequences, the proteins derived from these pan-edited cryptogenes will evolve almost twice as fast as the proteins encoded by non-edited genes [47•]. This is a surprising finding, as the necessity for a coordinated evolution of the pre-edited mRNA and the corresponding gRNA might be expected to slow down the evolution of cryptogenes as compared to non-edited genes.

## RNA editing: ancient or derived?

The drastic differences in mini-circle and gRNA complexity and the extent of editing of homologous cryptogenes observed within the trypanosomatid family indicate that the editing system undergoes evolutionary changes that are more dramatic than the accumulation of point mutations [35••,36••,48]. Two major character states may be distinguished in regard to the extent of editing: pan-editing, with multiple overlapping gRNAs and a high minicircle complexity, as in *T. brucei*, and 5′- or internal editing, with low gRNA and minicircle complexity, as in *L. tarentolae*. The issue of the direction of evolution of the editing system in terms of these character states has been addressed in papers by Landweber and Gilbert [35••] and Maslov *et al.* [36••]. The distribution of pan-editing and 5′-editing of the COIII, ND7, and maxicircle unassigned reading frame 4 (MURF4; possibly a highly diverged ATPase subunit 6 [5]) cryptogenes in different lineages of trypanosomatids has been analyzed.

Using a rooted phylogenetic tree, it was shown that pan-editing occurs mainly in the early evolved lineages, whereas 5′-editing occurs in the lineages that appeared later in evolution. It is hypothesized [30,32,36••,48] that, in the course of evolution, pan-edited cryptogenes were substituted with less edited homologs by a mechanism that involved reverse transcription of partially or completely edited mRNAs and homologous recombination (Fig. 2). It is also suggested [32,36••] that these substitution events are selected for if they are accompanied by a loss of one or more minicircle classes specifying the corresponding gRNAs. Such a loss would impair editing in cells that relied on the original pan-edited cryptogenes and would most likely be lethal for these cells, whereas cells with maxicircle molecules containing substitutions would survive. The retention of a limited amount of editing at the 5′ end of a domain may be required for regulation of mitochondrial biosynthetic activity during the parasite life cycle. In some cases, such as the COIII gene in *Blastocrithidia culicis*, and possibly also the ND1, ND4, ND5, and COI genes in all trypanosomatids, the original pan-edited cryptogenes are replaced with fully edited genes [36••].

## Complex gRNA repertoire and lack of network rotation are ancient characters

It has been known for some time that minicircle populations in the kinetoplast DNA network undergo changes during growth of the cells in culture [49], which corresponds to the insect vector stage of the life cycle. We have demonstrated recently a loss of minicircles specifying gRNAs required for editing of ND8, ND9, G3, G4, and ND3 in an old laboratory strain of *L. tarentolae* [46]. The lack of editing of ND8 and ND9 has also been observed in an old culture strain of *Crithidia fasciculata*

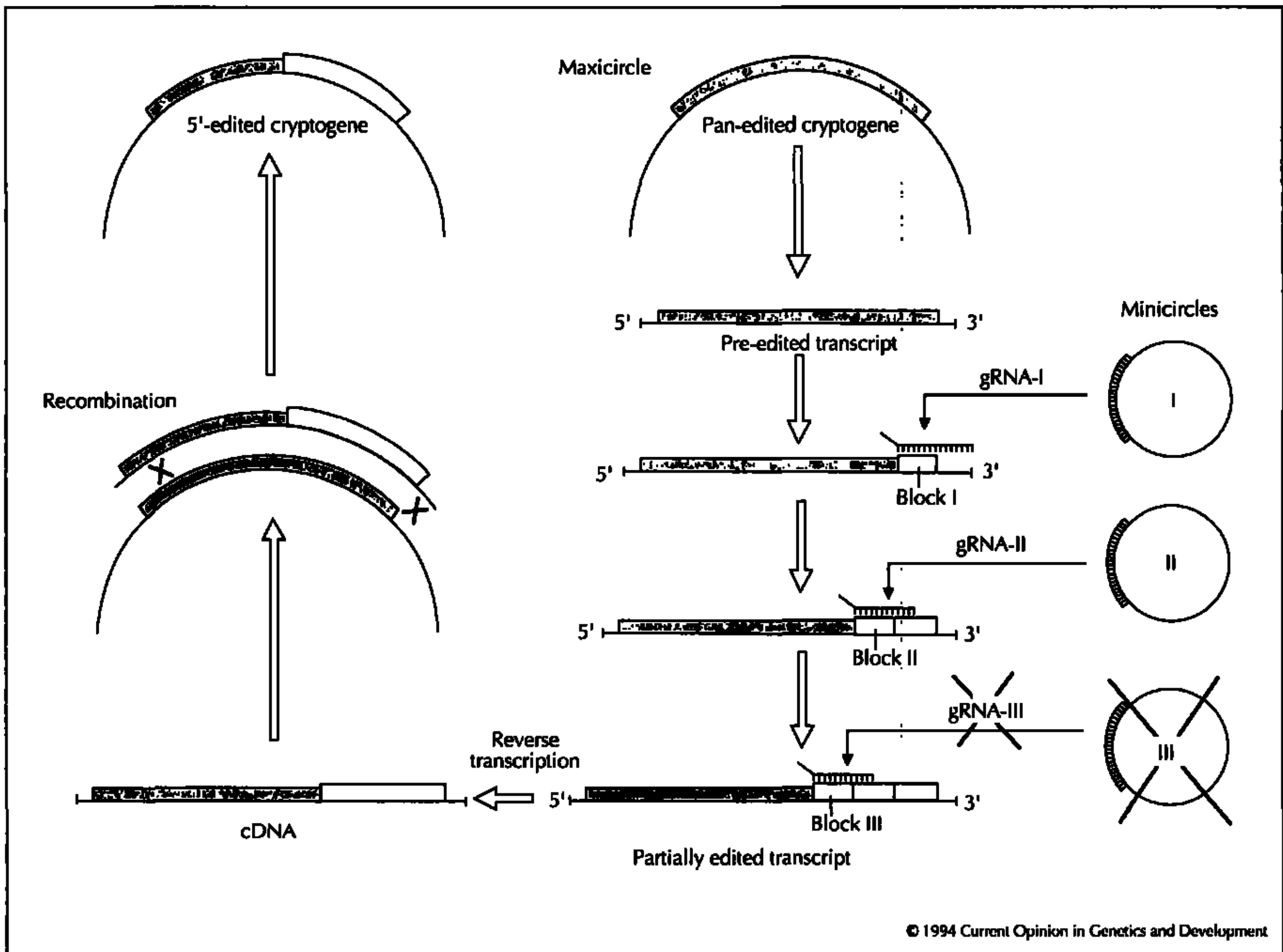


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**Fig. 2.** A model for the evolution of editing in kinetoplastid protozoa. The primary transcript (thick tinted line) is edited by the first three overlapping gRNAs. Edited sequences are indicated by open boxes. A cDNA for a partially edited transcript replaces the original cryptogene in one of the maxicircles by homologous recombination. If the minicircle class encoding one of the three gRNAs is lost (in this example, shown as a cross for gRNA-III), cells lacking the substituted cryptogene could not edit this transcript. Cells with a substituted cryptogene would survive the loss of the gRNA, as the uridylates would already be genomically encoded. Modified from [32].

[50•]. The protein products of these genes may not be required under culture conditions, thus removing the selective pressure to maintain complete sets of gRNAs. In the venereally transmitted parasite *Trypanosoma equiperdum*, which has extensive deletion and rearrangement of the maxicircle genome and is therefore incapable of growth in the insect vector or as the procyclic form in culture, the transcripts of several pan-edited cryptogenes are present but are not edited, as the result of the absence of gRNAs caused by the loss of all but a single minicircle class [51•].

The mechanism of the loss of minicircle sequence classes by growth in culture or during evolution has been proposed to be the result of missegregation of low copy number minicircles at network division [30,32,36••]. Minicircles are detached from the network in S phase by a topoisomerase II and recatenated after replication from two 'replisomes' situated at opposite ends of the network [52••,53–55]. This is followed in early G2 by the covalent closure of all replicated circles and the splitting of the doubled network into two progeny networks that segregate into the daughter mitochondria. In *T. brucei*, the network appears to remain stationary and the minicircles are recatenated at one of the two antipodal sites [56•]. This would appear to favor the missegregation of low copy number minicircles. Because of the extremely large genomic complexity of the minicircle DNA and the existence of redundant gRNA genes, however, this loss of minicircles could very well not disrupt editing. The evolution of editing in the trypanosomatids involved both a loss of pan-edited cryptogenes and a simplification of the minicircle-encoded gRNA repertoire. In later evolved lineages such as *Crithidia*, perhaps as a mechanism to reduce the missegregation of minicircles in daughter networks, the kinetoplast DNA network actually rotates as the daughter minicircles are sequentially recatenated [52••], thereby distributing the minicircles around the periphery of the network more randomly. We hypothesize that a large genomic complexity of minicircle DNA and the resultant gRNA redundancy, as well as the lack of rotation of the network, are ancestral characters.

## Editing and splicing: offsprings from the same root?

The conclusion that pan-editing is a primitive state leads us to suggest that pan-editing and editing itself are at least as ancient as the trypanosomatid family. Our recent discovery of pan-editing and gRNAs in the mitochondrial genome of *Trypanoplasma borreli*, a cryptobiid kinetoplastid, pushes editing back to an ancestor of the entire kinetoplastid lineage [57]. Estimates of genetic divergence indicate that the ancestral kinetoplastid existed possibly as early as 500 million years ago [34••]. This conclusion gives more creditability to speculations that editing and splicing have a common origin in an RNA world, a hypothesis devised on the basis of the similarity between RNA splicing and the transesterification model of RNA editing [19,20]. An alternative enzymatic model [11,18] is also plausible at this time, however, and in this model, editing would have no clear evolutionary relationship with RNA splicing.

Recent evidence that editing and splicing may have evolved from the same primordial genetic mechanism has been provided by the demonstration that a Group II self-splicing intron lariat can perform RNA editing like reactions *in vitro* [58•]. As in RNA editing, the internal polymerization site is determined by intermolecular base-pairing and nucleotides are added in a 3′ to 5′ direction; however, base-pairing in this model system occurs 5′ of the editing site instead of 3′ and the source of the added nucleotides is the mRNA itself.

It is clear that only a dissection of the actual mechanism of RNA editing can settle this issue.

## Mechanism of RNA editing: an unsolved problem

Several enzymes that are involved potentially in RNA editing [an editing site specific endonuclease, a terminal uridylyl transferase (TUTase), and an RNA ligase] are present in mitochondrial extracts from trypanosomatids [18,59–61]. In addition, a gRNA/mRNA chimera forming activity has been demonstrated in both *T. brucei* [62,63] and *L. tarentolae* [20]. A possible clue for the role of these activities in RNA editing lies in the detailed biochemical characterization of ribonucleoprotein (RNP) complexes in these mitochondrial extracts. Two major types of gRNA-containing native RNP complexes have been characterized recently in *T. brucei* and *L. tarentolae*. In *T. brucei*, a 19S complex I was shown to contain gRNA, TUTase, RNA ligase, and a chimera-forming activity, and a 35S complex II was shown to contain gRNA, RNA ligase, and a chimera-forming activity, but little (if any) TUTase, as detected on glycerol gradients [64]. In *L. tarentolae*, as demonstrated by glycerol gradient centrifugation and native gel analysis, a 10–13S set of approximately six 'T-complexes' was shown to contain gRNA, TUTase, and several proteins and could be self-labeled with $^{32}$P-UTP, and a 25S 'G-complex' was shown to contain gRNA, proteins, and an *in vitro* internal U-incorporation activity [65••]. Native gel retardation experiments have shown that the RNP complexes can be detected also by incubation of the extracts with labeled exogenous synthetic gRNA or mRNA (F Bringaud, E Byrne, L Simpson, unpublished data). In *T. brucei*, four complexes, labeled G1–G4, have been detected with exogenous labeled gRNA, and the formation of these complexes is facilitated by some structural features of the gRNA and several specific proteins [66•,67•]. The available data do not allow a correlation of the G1–G4 complexes with the previously described 19S complex I and 35S complex II.

It is clear that the characterization of these RNP complexes in two trypanosomatid species is in its initial stages and no conclusions can yet be drawn regarding the mechanism of RNA editing.

## The polarity of RNA editing

The alternative models of random editing or misediting by misguiding as the cause of the overall 3′ to 5′ polarity of editing and the occasional incorrectly edited junction regions have been discussed in this review. On the basis of the finding of a high frequency of 3′ truncated gRNAs in amplified and cloned gRNA/mRNA chimeric molecules from *C. fasciculata*, Arts *et al.* [68•] have proposed that the apparent strict polarity of editing may be an artifact resulting from the creation of partially edited RNAs by truncated gRNAs. They argue that a flexibility exists in the editing process that is more in line with models that do not invoke a strict 3′ to 5′ polarity [68•,69].

## Conclusions

RNA editing in kinetoplastid mitochondria by a multiple overlapping gRNA mediated mechanism appears to have arisen early in the evolution of eukaryotes, but the precise time and lineage remain unclear. The alternative scenarios of a truly ancient origin in the RNA world, or a derived origin occurring in the lineage of kinetoplastid flagellates or in the eubacterial endosymbiont that became the protomitochondrion, cannot be distinguished with the current data. A clearer picture may develop when the precise biochemical mechanism is understood and after a more complete survey of the presence or absence of editing in phylogenetically significant organisms.

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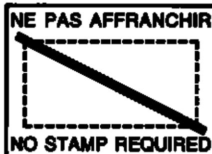


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