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Review

Uridine insertion/deletion RNA editing in trypanosome mitochondria — a review

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Abstract

The uridine insertion/deletion RNA editing in trypanosome mitochondria is a unique posttranscriptional RNA maturation process that involves the addition or removal of uridine residues at precise sites usually within the coding regions of mitochondrial transcripts. This process creates initiation and termination codons, corrects frameshifts and even builds entire open-reading frames from nonsense sequences. The development of several in-vitro editing assays has provided much insight into the molecular mechanism of RNA editing, which appears to involve cleavage, U addition, exonuclease trimming and ligation, essentially as proposed in the original 'enzyme cascade' model (Blum, B., Bakalara, N., Simpson, L., 1990. A model for RNA editing in kinetoplastid mitochondria: 'Guide' RNA molecules transcribed from maxicircle DNA provide the edited information. Cell 60, 189–198). However, little is known about the biochemical properties of the proteins involved and the significance and role of this process. This article is a review of recent findings on uridine-insertion/deletion editing in trypanosome mitochondria, with an emphasis on the proteins isolated and characterized that may have a role in this process.

Keywords: Kinetoplast DNA; *L. tarentolae*; Mitochondria; RNA editing; *T. brucei*

1. Introduction

The trypanosomatids represent a major group of parasitic protozoa within the kinetoplastid flagellates. They include several pathogenic species from the genera *Leishmania* and *Trypanosoma*, which infect a wide range of hosts, including domestic livestock and man. Probably due to the early divergence of this eukaryotic lineage in evolution (Fernandes et al., 1993), studies of the molecular biology of these cells have produced a plethora of novel and significant discoveries, which include polycistronic transcription (Clayton, 1992), *trans*-splicing of precursor mRNAs (Graham, 1995), glycosylphosphatidylinositol anchors of membrane proteins (McConville et al., 1992), antigenic variation (Pays et al., 1994), and the presence of a unique organelle containing most of the glycolytic enzymes (Opperdoes et al., 1984). However, the most remarkable of these unusual phenomena are found within the single tubular mitochondrion, namely the organization of the mitochondrial DNA into a network of catenated circles (Simpson, 1987; Shapiro and Englund, 1995) and the uridine insertion/deletion editing of mitochondrial mRNAs. This article provides an overview of the latest findings on RNA editing in trypanosome mitochondria.

Abbreviations: CYB, cytochrome b mRNA; GDH, glutamate dehydrogenase; gRNA, guide RNA; kDNA, kinetoplast DNA; ND7, NADH dehydrogenase subunit 7 mRNA; TUTase, terminal uridylyl transferase; U, uridine.

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2. Messenger RNA editing creates translatable messages

The mitochondrion of the trypanosomatid kinetoplastid protozoa is organized into a disk-shaped region

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adjacent to the basal body of the flagellum and one or more tubular projections (Paulin, 1975, 1977, 1983; Simpson and Kretzer, 1997). The former is termed the kinetoplast region since it contains the mitochondrial or kinetoplast DNA (kDNA) in the form of a giant network of thousands of catenated DNA circles (Simpson, 1987; Shapiro and Englund, 1995). There are approximately 10 000 catenated minicircles and 50 catenated maxicircles per network. The maxicircles are the functional equivalent of conventional mitochondrial DNAs as they encode ribosomal RNAs and some of the proteins involved in the mitochondrial respiratory chain and oxidative phosphorylation. It was evident upon sequencing the maxicircle from *Trypanosoma brucei*, *Leishmania tarentolae* and *Crithidia fasciculata* that several genes had frameshifts and lacked initiation or termination codons. Other regions in *T. brucei* that encoded recognizable genes in *L. tarentolae* and *C. fasciculata* contained no open-reading frames at all (Simpson et al., 1987). The study of this very unusual gene organization led to the discovery of RNA editing (Benne et al., 1986). This process involves the insertion or deletion of uridine (U) residues at precise sites, usually within coding regions, of approximately 12 of the 18 mRNA transcripts of the maxicircle molecule (Feagin et al., 1988b; Shaw et al., 1988, 1989). These insertion/deletion events create methionine translation initiation codons, correct frameshifts and even create entire open-reading frames (Feagin et al., 1988a; Maslov et al., 1992; Alfonzo et al., 1997).

The genetic function of minicircles was a mystery for many years until the discovery of a novel class of small RNA molecules that were named guide RNAs (gRNAs) (Blum et al., 1990). In *L. tarentolae*, these gRNAs are transcribed from both the maxicircles and the thousands of catenated minicircle components of the kDNA (Sturm and Simpson, 1990a), and they contain the sequence information that the editing machinery needs for the correct insertion or deletion of Us at precise sites (Blum et al., 1990). The gRNAs have a distinctive primary structure (Fig. 1). The 5′-most sequence, or anchor, is complementary to the mRNA just 3′ of the first editing site and provides the specific interaction between the gRNA and its cognate mRNA. The central portion of the gRNA contains the editing information and is complementary to mature edited mRNA sequence, allowing G–U non-canonical base pairs. An oligo[U] tail is added at the 3′ end of the gRNAs posttranscriptionally (Blum and Simpson, 1990). The function of this tail is not known, but it might interact with purine-rich regions upstream of the editing region (Blum et al., 1990; Seiwert et al., 1996).

Editing generally proceeds from 3′ to 5′ both within a single gRNA-mediated 'block' (Sturm and Simpson, 1990b) and also within the entire editing 'domain', although a certain amount of non-progressive editing of

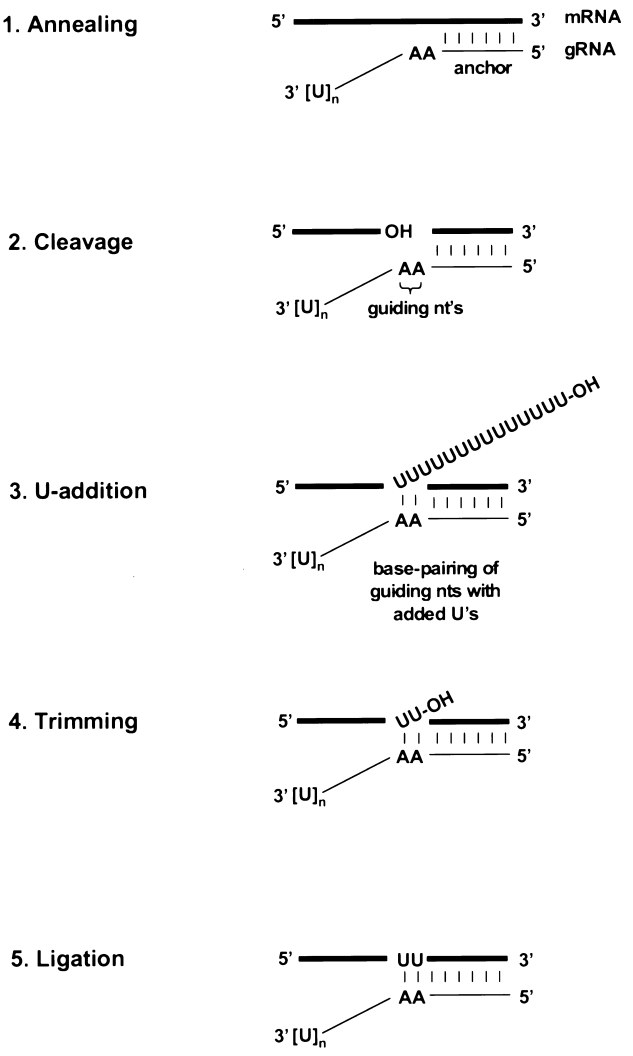

Fig. 1. Model for U-insertion RNA editing (Alfonzo et al., 1997). A thick line represents the mRNA molecule, and a thin line represents the cognate gRNA. Vertical lines indicate base pairs. The 3′ oligo[U] tail in the gRNA is represented as an overhang, but it is known to interact with the pre-edited region of the mRNA (Leung and Koslowsky, 1999). U-deletion editing involves a substrate mRNA with one or more Us at the 3′ end of the 5′ cleavage fragment and an absence of guiding nucleotides in the gRNA to base pair with these Us, which are then trimmed by the exonuclease activity. See Section 5 for detailed information about the enzymes involved in each step.

cDNAs has been reported in *T. brucei* (Decker and Sollner-Webb, 1990; Koslowsky et al., 1991; Militello and Read, 1999) and *L. tarentolae* (Sturm and Simpson, 1990b; Maslov et al., 1992). The overall 3′ to 5′ polarity (Abraham et al., 1988) within a domain is due to the creation of new anchor sequences by the downstream gRNA for hybridization of the adjacent upstream gRNA (Abraham et al., 1988; Maslov and Simpson, 1992). A consequence of this polarity is that in the steady-state population of a given mRNA that undergoes editing, both the unedited and edited versions are detected as well as partially edited intermediates.

The uridine insertion/deletion editing in trypanosome mitochondria seems to follow an 'enzyme cascade' mechanism (Blum et al., 1990; Kable et al., 1996; Seiwert et al., 1996; Alfonzo et al., 1997). This model (Fig. 1) proposes that the gRNA first hybridizes downstream of the first editing site via the anchor. Then, a specific endonuclease cleaves at the mismatched base. A terminal uridylyl transferase activity (TUTase) adds U residues to the 3′ terminus of the 5′ cleavage fragment. The added U residues can then base-pair with the guiding nucleotides in the gRNA and extend the duplex. A 3′ to 5′ exonuclease trims back the non-base-paired Us, and an RNA ligase joins the modified cleavage fragments. The editing machinery then proceeds to the next upstream site. U deletions are proposed to occur by the same mechanism, but non-base-paired Us on the 3′ end of the 5′cleavage fragment are removed by an exonuclease prior to religation of the fragments. An alternative mechanism for editing, the transesterification model, was also proposed upon the discovery of gRNAs (Blum et al., 1991; Cech, 1991). This model suggested that the editing reaction occurred by a series of reactions analogous to splicing and was supported by the detection of chimeric gRNA–mRNA molecules in vivo. Nevertheless, data from in-vitro editing reactions argue against the transesterification model and strongly support the enzyme cascade model (see Section 4).

3. Extent and evolution of RNA editing

The number of Us added or deleted by RNA editing varies between mRNAs in the same organism. Some mRNAs are edited only at their 5′ or internal regions, whereas others are edited throughout the gene, a phenomenon named 'pan-editing' (Simpson and Shaw, 1989). The extent of editing within a given gene may be different between species. For example, a transcript that is pan-edited in one organism might be only 5′-edited in another. It has been proposed that ancestral cryptogenes were pan-edited and were replaced by partially edited genes by a retroposition mechanism by which cDNA copies of partially edited mRNAs replaced the original pan-edited cryptogenes (Landweber, 1992; Simpson and Maslov, 1994a). This led to the creation of 5′-edited genes or even completely edited genes. It was proposed that the loss of specific minicircle-encoded gRNAs occurred by random missegregation of minicircles at cell division (Simpson and Maslov, 1994a). This hypothesis is supported by the finding that such a loss was detected in a laboratory strain of *L. tarentolae* after prolonged culture (Thiemann et al., 1994). It has been suggested that RNA editing may have arisen as an adaptation to a parasitic lifestyle (Simpson and Maslov, 1994a). However, editing has also been detected in *Bodo saltans* (Blom et al., 1998), a free-living protist belonging to a sister kinetoplastid group, the bodonids. Since the bodonids appear to represent the ancestors of the trypanosomatids (Simpson and Maslov, 1994b), it is possible that editing arose in evolution before parasitism (Blom et al., 1998) and is an ancient process that existed more than 500 million years ago (Fernandes et al., 1993; Simpson and Maslov, 1994b)

RNA editing is also regulated in the life cycle of *T. brucei*. This trypanosome alternates between mammalian and insect hosts and must change its metabolism to adapt to the different environments it encounters. One of the most striking of these changes is observed in the mitochondrion. In the insect host midgut (procyclic form), the cells possess a fully operational mitochondrion with a functional Krebs cycle and cytochrome-based respiration. In the mammalian host (bloodstream forms), the production of energy relies on the oxidation of glucose to pyruvate, and mitochondrial respiratory function is repressed (Priest and Hajduk, 1994). Since editing corrects the messages coding for some of the components of the mitochondrial respiratory chain, it is not surprising that important changes in the editing of several mRNAs are detected between the bloodstream and the procyclic forms (Feagin and Stuart, 1988; Koslowsky et al., 1990).

4. In-vitro RNA editing assays

The development of in-vitro editing systems has been extremely useful for understanding the editing reaction. Both gRNA-independent and gRNA-dependent in-vitro activities have been reported. A gRNA-independent U-insertion editing-like activity was observed with two substrates, *CYB* and *ND7*, using mitochondrial extracts from *L. tarentolae*. A primer extension assay was used to follow insertions at a single editing site, and it was observed in the case of the *CYB* mRNA substrate that there was a requirement for the formation of a short RNA duplex with sequences upstream and downstream of the editing domain (Connell et al., 1997). In a recent report (Brown et al., 1999), a novel gRNA-independent editing assay was developed that followed the insertion of Us into a circular *CYB* RNA substrate. Using this assay, the authors showed that a 34-nucleotide AU-rich sequence element located just upstream of the editing domain was responsible for the U-insertion activity and that at least one protein specifically interacted with this U-rich sequence. The mechanism that confines the U-insertions to the preedited region in the absence of exogenous or endogenous gRNAs is not understood, nor is the possible physiological relevance of the gRNA-independent U-insertion activity, although it has been proposed to act as an initiation complex for the correct assembly of the gRNA-dependent editing machinery (Connell et al., 1997).

gRNA-dependent in-vitro editing assays, both U-insertional and U-deletional, have also been reported. The *T. brucei* system involves the editing of sites 1 and 2 of the A6 mRNA. U deletions normally occur in site 1 and U insertions at site 2. A cognate synthetic gRNA supplied *in trans* was shown to mediate either U deletions at site 1 (Seiwert and Stuart, 1994; Cruz-Reyes and Sollner-Webb, 1996; Seiwert et al., 1996) or U-insertions at site 2 (Kable et al., 1996). The editing efficiency of the *T. brucei* system is low (approximately 2%) but is sufficient to be able to directly detect intermediates by gel analysis. Both the predicted 5′ and 3′cleavage fragments were detected with synthetic end-labeled mRNA substrates, and Us were shown to be added to the 3′ end of the 5′ cleavage fragment as predicted by the enzyme cascade model. gRNA–mRNA chimeras were also detected, but these appeared in a time-course experiment after the cleavage fragments, suggesting that the chimeric molecules were by-products of the editing reaction (Seiwert et al., 1996). In support of this, stabilization of the interaction of the 5′ cleavage fragment and the 3′ end of the gRNA by the creation of artificial base pairs decreased the abundance of chimeras without greatly affecting the formation of the mature edited product (Seiwert et al., 1996). However, blocking the 3′ end of the gRNA by periodation eliminated editing, suggesting an as-yet unclear role for the 3′ end of the gRNA in this system (Seiwert et al., 1996). In a recent paper, Burgess et al. (1999) have shown that mutations in the gRNA that increase the base-pairing interaction between the 3′ end of the gRNA and the mRNA remove the periodate-induced inhibition of editing, and they suggested that the 3′ end of the gRNA may bind a protein that stabilizes the gRNA–mRNA interaction.

The *L. tarentolae* system involved the detection of U insertions at site 1 of the *ND7* mRNA (Fig. 2). This in-vitro reaction was less efficient than the *T. brucei* reaction, and the analysis required an RT-PCR step (Byrne et al., 1996). Both gRNA-independent and gRNA-dependent U insertions were detected, depending both on the length of the substrate RNA and on the presence of a cognate gRNA supplied in trans. In addition to predominant gRNA-guided U insertions, a background ladder of up to 13 gRNA-dependent unguided U insertions was also observed. It was speculated that this ladder represented a low level of misediting perhaps due to religation prior to complete trimming of the 3′ end of the 5′ cleavage fragment, and that this may also occur in vivo. The key result from the *L. tarentolae* system was that blocking of the 3′ end of the gRNA by periodation had no effect on in-vitro editing, thereby eliminating the transesterification model, which proposed a transfer of Us from the 3′ end of the gRNA to the editing site (Byrne et al., 1996).

5. Mechanism of editing and proteins involved in editing

5.1. mRNA–gRNA hybridization and gRNA-binding proteins

The initial event in the editing reaction is a hybridization of the 5′ end of the gRNA to the mRNA just downstream of the first editing site to form an anchor duplex. Additional stabilizing interactions may come from the 3′ oligo[U] tail present in all gRNAs. It has been proposed that this U tail binds to purine-rich regions upstream of the editing sites, thereby stabilizing the gRNA–mRNA interaction and preventing the mRNA 5′ cleavage fragment from leaving the editing complex (Blum and Simpson, 1990). This role is supported by the observation that removal of the oligo[U] tail inhibits the production of the edited product in the *T. brucei* in-vitro U deletion assay but has no effect on the initial cleavage reaction (Seiwert et al., 1996). This inhibition was reversed by stabilizing the interaction between the gRNA 3′ end and the mRNA sequence upstream of the editing site (Burgess et al., 1999).

In addition, it has recently been shown, by mapping the contacts between gRNA and mRNA using cross-linking agents (Leung and Koslowsky, 1999), that the gRNA 3′ oligo[U] tail binds purine-rich sequences five to 28 bases upstream of the first editing site in the *T. brucei* system. The predicted secondary structure for gRNA–mRNA interactions described in the same report suggests that the U tail not only stabilizes the RNA interactions but can also eliminate mRNA secondary structure and thereby increase the accessibility of the editing activity to the editing sites.

However, in a recent study of the *L. tarentolae* in-vitro editing system utilizing a cognate gRNA provided in cis at the 3′ end of the mRNA (Fig. 3), Kapushoc and Simpson (1999) showed that the absence of the 3′ oligo[U] tail does not have any effect on U-insertion editing. Nevertheless, they also showed that the presence of artificial base pairs between the 5′ cleavage fragment and the 3′ end of the gRNA had a dramatic beneficial effect on the U-insertion activity (Fig. 3 and Fig. 4). These observations suggest that retention of the 5′ cleavage fragment within the editing complex requires the presence of specific protein-protein and/or protein-RNA interactions in vivo that cannot be efficiently reproduced in vitro and that these interactions can be mimicked by providing a gRNA with a artificially stabilized 3′end (Kapushoc and Simpson, 1999). Similar results were reported for the in-vitro U insertion assay in *T. brucei* (Burgess et al., 1999).

Given the extensive interactions between the macromolecules involved in editing, it is likely that the reaction requires not only components actively involved in catalyzing the information transfer but also structural proteins dealing with positioning, unwinding or assisting in

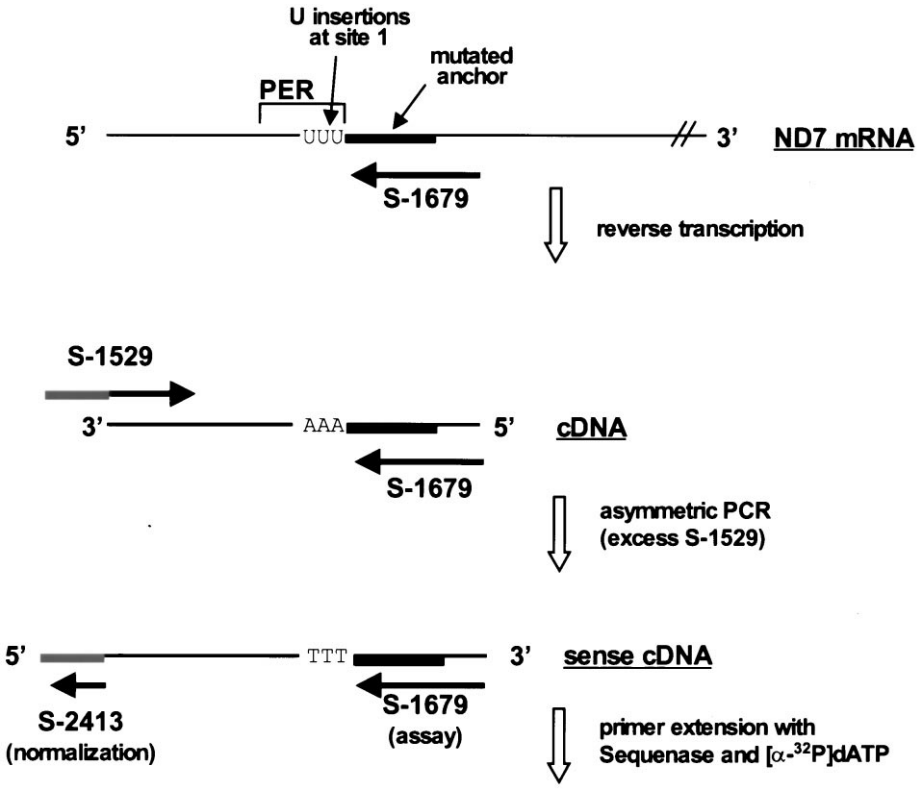

Fig. 2. Primer extension assay for in-vitro editing with *L. tarentolae ND7* mRNA as a substrate (Byrne et al., 1996). *ND7* mRNA is edited in two domains (Shaw et al., 1988, 1989). The 5′ domain has seven editing sites, and the mature edited mRNA has an insertion of seven Us in site 1, which is mediated by seven guiding nucleotides in the cognate gRNA; the assay monitors in-vitro U insertions at site 1. The mRNA has mutations in the anchor sequence that prevent the endogenous gRNA from base-pairing. The added gRNA has compensatory mutations in the anchor sequence. The number of guiding nucleotides in the synthetic gRNAs varied from three to seven. From Kapushoc and Simpson (1999), with permission.

the annealing of the gRNA and the mRNA. A possible target for such structural proteins is the gRNA, as it plays a central role in the editing process, and indeed, there is clear evidence for gRNA-binding proteins in different organisms (Read et al., 1994a; Byrne et al., 1995; Leegwater et al., 1995). Candidates that have been recently identified and characterized include the *T. brucei* gBP21, TBRGG1 and RBP16 proteins and the *L. tarentolae* glutamate dehydrogenase.

gBP21 was identified in *T. brucei* mitochondria as an arginine-rich 21 kDa protein that bound to several different gRNAs (Koller et al., 1997). Despite the fact that no RNA-binding motif could be identified in the primary amino acid sequence, the affinity of this protein for gRNA is very high, with a K_d of 10 nM. The recognition seemed to be independent either of primary sequence or the oligo[U] tail, and most likely involves the secondary structure motifs that seem to be common to all gRNAs (Schmid et al., 1995). The binding of gBP21 to gRNA resulted in a increased stability of the gRNA, which suggested a role in the initial events leading to the assembly of the RNA editing machinery (Koller et al., 1997). Moreover, gBP21 is physically associated with active RNA editing complexes (Allen et al., 1998), and an antiserum raised against this protein was able to inhibit the in-vitro editing reaction (Lambert et al., 1999). In addition, a *T. brucei* mitochondrial extract immunodepleted of gBP21 no longer exhibited editing activity (Lambert et al., 1999). However, a cell line in which both alleles of the *gBP21* gene were knocked out was still able to edit mRNAs and showed a normal gRNA pool (Lambert et al., 1999). These knockout results argue against a role of gBP21 in editing. Nevertheless, a negative result with gene-knockout experiments is difficult to interpret since there is always the possibility of the existence of redundant genes, and molecular redundancy has been reported to occur in various biochemical systems (Thomas, 1993). The role of gBP21 is therefore unclear, but this protein might be involved in mitochondrial transcription or RNA turnover since, in the double knockout cell line, the abundance of mitochondrial mRNA transcripts was reduced (Lambert et al., 1999), and these cells were only viable as bloodstream forms in which the mitochondrial function is dispensable (Stuart and Gelvin, 1982).

Another gRNA-binding protein characterized recently is the *L. tarentolae* mitochondrial glutamate

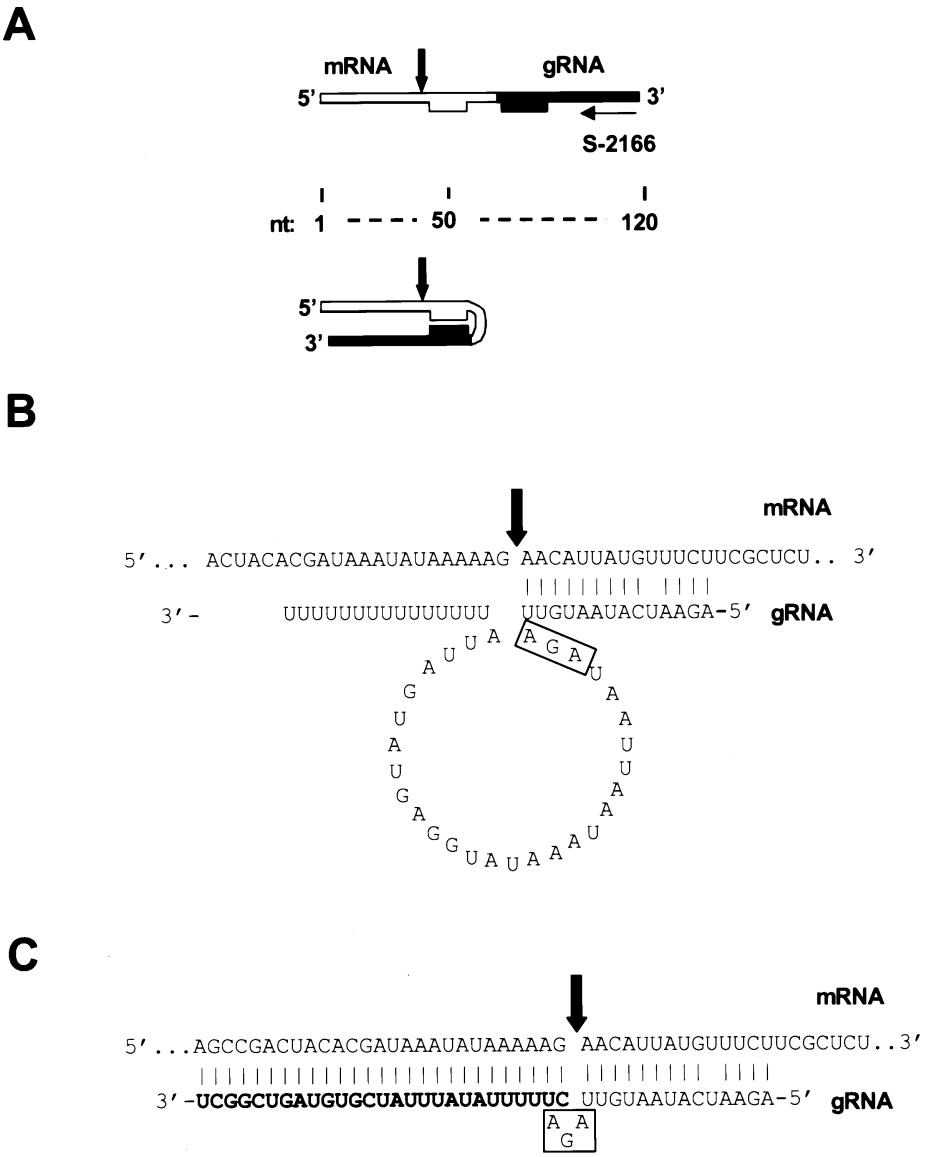

Fig. 3. U insertion RNA editing mediated by *cis*-acting gRNAs. (**A**) diagram of the *cis*-acting gRNA transcript used to study the role of the 3′ end of the gRNA in the editing reaction (Kapushoc and Simpson, 1999). Filled segment represents gRNA sequence, empty segment the mRNA sequence. The thicker portion represents the anchor sequence and vertical arrows indicate editing site 1. The proposed folding to form an intramolecular anchor duplex is also shown. (**B**) Anchor hybridization of the mutated anchor transcripts (top) and the gRNA with a stabilized 3′ end (bottom). Only the relevant portion of the mRNA is shown. Arrows indicate editing site 1. The three guiding nucleotides for editing site 1 are boxed in the gRNA sequences. (**C**) The bases that have been substituted in the gRNA sequence to form a stable 3′ end are shown in bold. From Kapushoc and Simpson (1999), with permission of Cambridge University Press.

dehydrogenase (GDH) (Bringaud et al., 1997). This 110 kDa protein was shown to bind to the 3′ oligo[U] tail of gRNA. The interaction apparently involved the NADP(H)-binding pocket of the enzyme since the binding could be specifically competed with this dinucleotide. In addition, GDH is also able to bind UTP, and this binding could be competed by NADP(H) (Bringaud et al., 1997). Since the *L. tarentolae* in-vitro editing activity was also inhibited by high concentrations of NADP(H), it was proposed that GDH might be involved in the editing reaction (Bringaud et al., 1997). This is an attractive possibility since it has been proposed that some metabolic enzymes that are able to bind RNA represent a novel class of proteins that may have a regulatory role linking metabolic and genetic functions (Hentze, 1994). This raised the possibility that *L. tarentolae* GDH could represent a link between mitochondrial metabolism and RNA editing (Bringaud et al., 1997). In a test of this model, the homologous *T. brucei GDH* gene was cloned and knocked out in bloodstream forms (Estévez et al., 1999), using a tetracycline-inducible conditional expression system (Wirtz et al., 1999; Wirtz and Clayton, 1995). Bloodstream forms were chosen for this study since editing of several mitochondrial genes

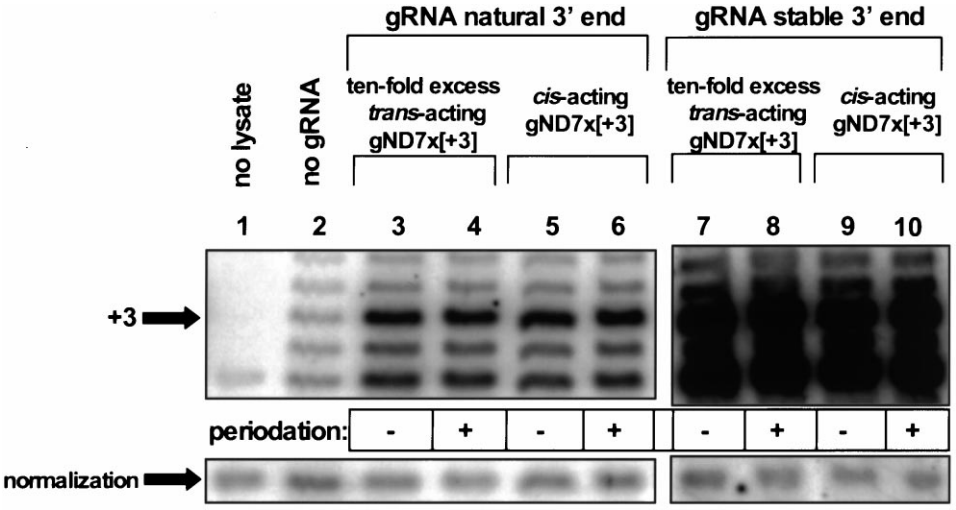

Fig. 4. Effect of alterations of the 3′ end of the gRNA and effect of stabilization of the gRNA–mRNA interaction on U insertions at site 1 of the synthetic *ND7* mRNA. Lanes 3–6, gRNAs with natural 3′ ends. Lanes 7–10, gRNAs with mutated 3′ ends as shown in Fig. 3C. The ND7 mRNA substrate was reacted with *trans*- and *cis*-acting gRNAs with and without periodate treatment, as indicated by periodation: + or −. Periodation treatment blocks the 3′ end of the gRNA. The normalization band below each lane is the primer extension signal from Us present in the 5′ PCR primer (see Fig. 2). From Kapushoc and Simpson (1999), with permission of Cambridge University Press.

occurs (Corell et al., 1994) but is dispensable for viability (Stuart and Gelvin, 1982). *GDH*-null mutants were viable, exhibited no apparent phenotypic changes and showed no differences in the relative abundance of edited mRNA for three different cryptogenes. The knockout results suggest that the gRNA-binding properties of GDH may not play a role in RNA editing. However, as in the case of gBP21, one cannot rule out the presence of redundant genes or another more subtle role in RNA editing, or the possibility that the protein may play a role in procyclic cells that cannot be detected in blood-stream cells.

Another putative gRNA-binding protein, TBRGG1, has been recently characterized in *T. brucei* (Vanhamme et al., 1998). This 75 kDa mitochondrial protein belongs to the RGG (Arg–Gly–Gly) family. It bound strongly to synthetic oligo[U], and it is probably one of the previously identified gRNA-binding proteins detected by UV-cross-linking in mitochondrial extracts of *C. fasciculata* (Leegwater et al., 1995) and *T. brucei* (Read et al., 1994a). This protein co-migrated with in-vitro editing activity in glycerol gradients, but no clear evidence was presented of a direct association of TBRGG1 with the editing machinery or with gRNAs. An antibody was raised against the recombinant protein, but the effect of immunodepletion of TBRGG1 on editing activity was not reported. Another protein of the RGG type, Nopp44/46 (Das et al., 1996), has also been described in *T. brucei*. It has a nucleolar localization, which suggests a role in rRNA metabolism. It is possible that TBRGG1 is involved in mitochondrial rRNA metabolism (Vanhamme et al., 1998), since trypanosome mitochondrial rRNAs are known to be polyuridylated at their 3′ end (Adler et al., 1991), and TBRGG1 is a oligo[U]-binding protein. This remains to be tested.

The third gRNA-binding protein characterized recently is the *T. brucei* RBP16 (Hayman and Read, 1999). This 16 kDa protein appeared to bind different gRNAs via the 3′ oligo[U] tail. This binding could be competed more than 50% with 1000-fold molar excess of unlabeled gRNAs in UV-cross-linking reactions. The protein contains a N-terminal cold-shock domain that represents a motif characteristic of the eukaryotic Y-box proteins and a C-terminal arginine- and glycine-rich region. Due to the presence of the cold-shock domain, RBP16 might play a role as an RNA chaperone in the course of the editing reaction, but this has not yet been tested. Antibodies raised against the recombinant protein were able to immunoprecipitate gRNAs and rRNAs, most probably due to the presence of the oligo[U] tail. As in the case of TBRGG1, this might suggest a role in mitochondrial rRNA metabolism. Functional knockouts of *TBRGG1* and *RPS16* as well as immunodepletion studies should give more insight into the role of these two proteins.

5.2. Cleavage of mRNA

The annealing of gRNA to the mRNA is followed by a specific cleavage in the mRNA at the mismatched base by an as-yet uncharacterized riboendonuclease. Several riboendonuclease activities has been detected in mitochondrial extracts from *T. brucei* and *L. tarentolae* (Harris et al., 1992; Michelotti et al., 1992; Simpson et al., 1992; Cruz-Reyes and Sollner-Webb, 1996; Piller et al., 1997). Three different riboendonuclease activities

have been described in a *T. brucei* mitochondrial extract (Piller et al., 1997), and one of these has the predicted features of a RNA editing riboendonuclease: it cleaves the mRNA immediately 5′ of a mRNA–gRNA duplex, and the cleavage is specific for the gRNA-directed editing site.

The only riboendonuclease isolated and characterized in detail from trypanosome mitochondria is MAR1 from *L. tarentolae* (Alfonzo et al., 1998). This nuclease is a 22 kDa mitochondrial protein that has a non-cleaved N-terminal mitochondrial targeting signal. Analysis of the enzymatic mechanism of MAR1 showed that cleavage of the pre-edited RNAs was independent of the addition of a cognate gRNA. In addition, the MAR1 enzyme did not distinguish between pre-edited and fully edited substrates. These facts suggest that this endonuclease may not be involved in editing. However, additional factors that were absent in the purified preparation could exist that confer gRNA-dependence on MAR1.

Cruz-Reyes et al. (1998) showed the existence of differences in adenosine nucleotide requirements for cleavage at U-deletion or U-insertion sites in the *T. brucei* system. Cleavage at a U-deletion site required high concentrations of ATP and ADP, whereas cleavage at a U-insertion site occurred in the absence of adenosine nucleotides, and it was even inhibited by high concentrations of these compounds. This effect appeared to be allosteric since ADP was not hydrolyzed in the reaction. At the 0.3 mM physiological concentration of ATP in the mitochondria of procyclic *T. brucei*, both U insertions and U deletion reactions occurred at half their maximal rates, suggesting that the RNA-editing reaction could be reasonably efficient in vivo (Cruz-Reyes et al., 1998). This phenomenon could be explained either by a single endonuclease that is interconverted between two states characterized by different preferences of cleavage at U insertion or U deletion sites, or by the existence of two different riboendonucleases. The physiological relevance of this phenomenon remains to be explained.

5.3. U insertions and U deletions

The next step in the editing process after the cleavage by the endoribonuclease is the addition of uridylate residues to the 3′-OH of the 5′ cleavage fragment by a 3′ terminal uridylyl transferase (TUTase) or the removal of non-base-paired U residues by a 3′ to 5′ riboexonuclease. TUTase activity has been detected in mitochondrial extracts and in more purified samples from *L. tarentolae* and *T. brucei* (Bakalara et al., 1989; Pollard et al., 1992; Peris et al., 1994; Rusche et al., 1997), and a 3′ exonuclease has been described in *T. brucei* mitochondrial extracts (Cruz-Reyes and Sollner-Webb, 1996) that is specific for U residues and generates 3′-OH termini, which are substrates for RNA ligase. We have recently succeeded in purifying to homogeneity a putative 3′ TUTase and also in detecting a U-specific 3′ to 5′ exonuclease from *L. tarentolae* mitochondria (R. Aphasizhev, M. Peris, L. Simpson, unpublished results).

5.4. Ligation

After U residues have been added or deleted, the two cleavage fragments are proposed to be ligated by an RNA ligase (Blum et al., 1990; Sabatini and Hajduk, 1995; Alfonzo et al., 1997). An RNA ligase activity has been detected in mitochondrial extracts from *L. tarentolae* and *T. brucei* (Bakalara et al., 1989; Pollard et al., 1992; Sabatini and Hajduk, 1995). This activity seems to be associated with two polypeptides of 50 and 45 kDa in both species (Peris et al., 1997) (described as 57 and 50 kDa in *T. brucei* (Sabatini and Hajduk, 1995; Rusche et al., 1997). These proteins can be adenylated by [α-^{32}P]ATP and deadenylated by RNA substrates. Blanc et al. (1999) partially purified an RNA ligase from *L. tarentolae* mitochondria and characterized the enzymatic activity. This enzyme requires α–β bond hydrolysis of ATP and has the predicted properties to play a role in the editing reaction since it is able to ligate two RNA fragments that are bridged by a complementary RNA (Fig. 5) and shows no specificity for the 3′ nucleotide of the 5′ cleavage fragment. The involvement of an RNA ligase in the editing reaction is also supported by the comigration in glycerol gradients of both U-insertion and U-deletion activities with RNA-ligase activity (Frech et al., 1995; Cruz-Reyes and Sollner-Webb, 1996) and by the observation that non-hydrolyzable α–β ATP analogs as well as pyrophosphate inhibited the editing reaction in vitro (Seiwert and Stuart, 1994; Cruz-Reyes and Sollner-Webb, 1996).

5.5. Additional reactions in the editing process

After the two cleavage fragments have been ligated, the entire process is then repeated at the next upstream editing site, resulting in the insertion or deletion of a precise number of Us and in an extension in length of the edited mRNA–gRNA duplex. Efficient processivity over multiple editing sites has not been demonstrated in vitro, and it is likely that there are additional factors involved in the process that have not yet been identified.

Finally, the unwinding of the gRNA-edited mRNA either to allow the next gRNA to hybridize or to bind to the ribosome may either involve breathing of the duplex due to an asymmetry of the weak G:U base pairs (Maslov and Simpson, 1992) and/or an RNA helicase (Missel and Göringer, 1994). Indeed, RNA helicase activity has been detected in mitochondrial extracts from *T. brucei* (Missel and Göringer, 1994) and a putative RNA helicase, mHEL61p, has been identified and the gene cloned (Missel et al., 1997). However, although

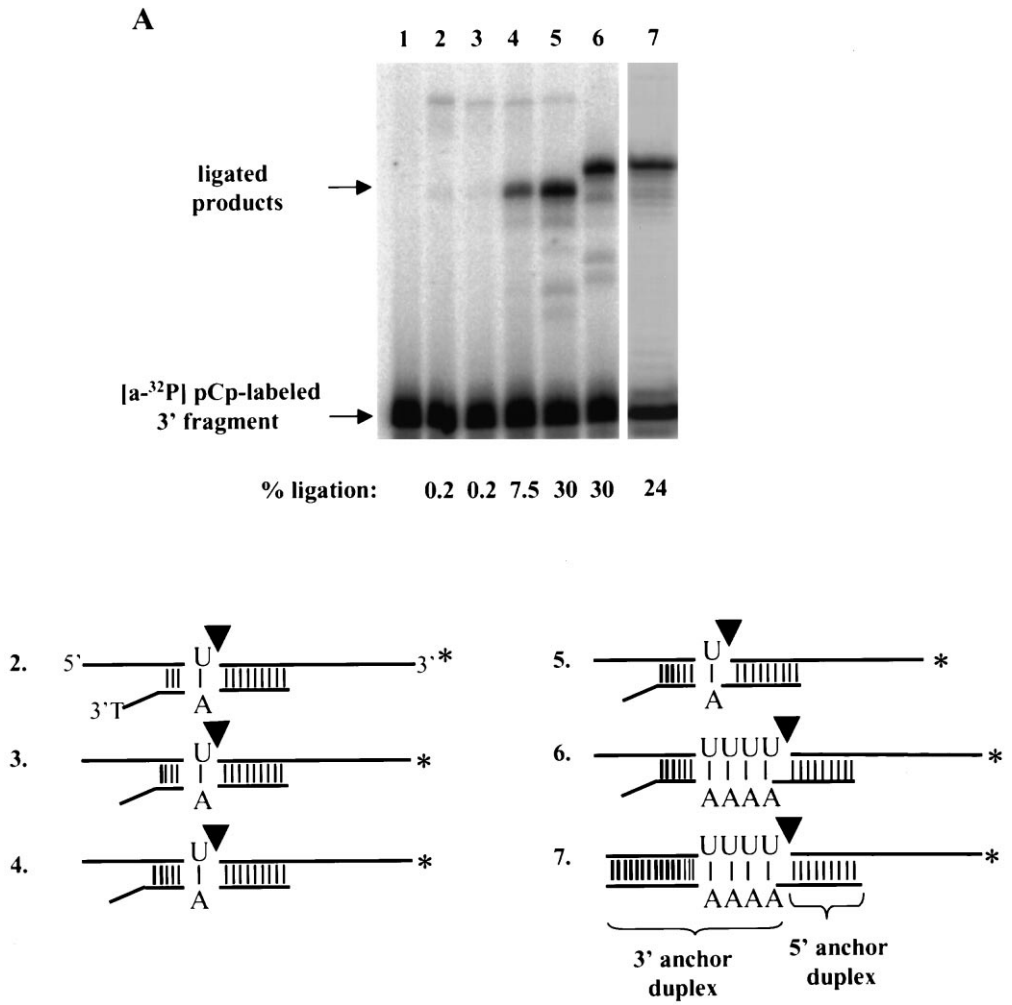

Fig. 5. Mitochondrial RNA ligase from *L. tarentolae* can efficiently ligate a nicked RNA substrate that is bridged by a complementary RNA. This figure shows the effect of varying the length of the 3′ duplex on the efficiency of bridged ligation. Lane 1, control with no bridge RNA. The mRNA 3′ cleavage fragment is labeled with [^{32}P]pCp at the 3′ end as indicated. The constructs for lanes 2–7 are shown diagrammatically below the gel. These constructs are based on the sequence of the *ND7* mRNA pre-edited region. A control ligation reaction in the absence of bridge RNA is in lane 1. The nick in each construct is indicated by an arrowhead. The length of the 3′ duplex varies from 4 to 19 bp. The base pairs added in constructs #3–7 as compared to construct #2 are indicated by bold lines. The synthetic 23 nt mRNA 3′ fragment was 3′-end-labeled with [α-^{32}P]pCp (*). A 3′ terminal dT nucleotide of the bridge RNA added during the synthesis is only shown in #2. The percentage ligation is the percentage of input substrate converted to product. From Blanc et al. (1999), with permission.

this protein contains a DEAD-box helicase motif, it did not fractionate with the *T. brucei* mitochondrial helicase activity. Moreover, gene disruption of the two alleles of the single copy *mHEL61p* gene led to the establishment of a cell line that exhibited no difference in RNA unwinding activity as compared to wild-type cells. This suggests that the observed mitochondrial helicase activity cannot be attributed to mHEL61p. Interestingly, the double knockout cell line showed a 70% reduction in the amount of edited RNAs products in vivo, whereas the unedited precursor and never-edited transcript levels were unaffected. Re-expression of the *mHEL61p* gene restored the levels of edited mRNAs (Fig. 6). In fact, this is the only gene knockout that has affected editing to date. Nevertheless, mitochondrial extracts obtained from the *mHEL61p*-null mutant were able to perform RNA editing in vitro. As we have mentioned before, the in-vitro editing assays are only able to detect editing at the first editing site, and it is therefore likely that mHEL61p does have a role in editing which is not monitored by the in-vitro assays available.

It has been proposed that RNA editing takes place in multiprotein complexes. In *T. brucei*, three different complexes, 10S, 20S and 35–40S, have been described (Pollard et al., 1992; Corell et al., 1996; Peris et al., 1997). The 10S complex contained TUTase and RNA ligase activities (Peris et al., 1997), and the 20S complex contained gRNAs, endonuclease, TUTase and RNA ligase (Corell et al., 1996). The 35–40S complex contained, in addition, pre-edited mRNAs (Pollard et al., 1992; Cruz-Reyes and Sollner-Webb, 1996). Purification of a 20S editing complex has been reported (Rusche

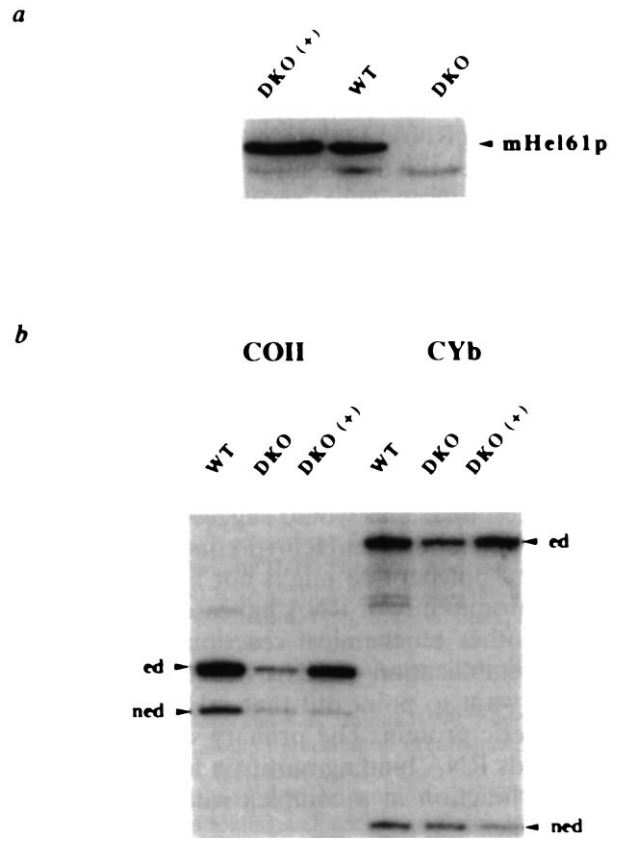

Fig. 6. Double-knockout of mHel61p gene from *T. brucei* procyclic cells causes reduced levels of edited mRNAs. Reexpression of mHel61p rescues the editing phenotype. (**a**) Western blot analysis of whole-cell extracts from wild-type trypanosome cells (WT), mHel61p double-knockout cells (DKO), and DKO trypanosomes reexpressing mHel61p [DKO (+)]. (**b**) Comparative primer extension analysis of COII and Cyb mRNAs from wild-type (WT) cells, mHel61p-minus cells (DKO), and double-knockout cells reexpressing mHel61p [DKO (+)]. From Missel et al. (1997), with permission.

et al., 1997). This complex contained a gRNA-dependent endonuclease, TUTase, exonuclease and RNA ligase activities. The exonuclease activity was U-specific (Rusche et al., 1997). The complex was shown to be composed of eight major polypeptides, three of which appeared to represent RNA ligase, based on their ability to autoadenylate. Both U-insertion and U-deletion activities were present in this complex. The proof that this complex represents a true editing complex must await purification and identification of the remaining polypeptides and reconstitution of editing activity with recombinant proteins.

A monoclonal antibody against the 35–40S complex was used to clone a novel gene coding for a 45 kDa protein, REAP-1 (Madison-Antenucci et al., 1998). The antibody inhibited in-vitro U-insertion editing, although a restoration of the editing activity by adding back recombinant REAP-1 was not reported. However, these data alone are not conclusive for the involvement of a protein in the editing reaction (as in the case of gBP21 described above), and the role of this protein in the editing reaction is still uncertain.

6. RNA editing and regulation of gene expression in the mitochondria

During the life cycle of *T. brucei*, many nuclear-encoded proteins show different patterns of developmental regulation. Genes are transcribed in long polycistronic units and processed by *trans*-splicing, and the individual processed transcripts may show a very different expression profile in different stages of the parasite. There is evidence that the regulation of gene expression is exerted mainly at the posttranscriptional level (Vanhamme and Pays, 1995).

The regulation of gene expression in the mitochondria of trypanosomes is poorly understood, and no promoters have been identified so far. Polycistronic transcripts have been detected, some of which are already edited, suggesting that editing occurs prior to cleavage into individual transcripts (Read et al., 1992; Koslowsky and Yahampath, 1997). Some mitochondrial transcripts, including those for rRNAs, show a differential abundance depending on the life-cycle stage (Feagin and Stuart, 1985; Michelotti and Hajduk, 1987; Michelotti et al., 1992). This regulation appears to be at a posttranscriptional level as in the nucleus (Michelotti and Hajduk, 1987; Michelotti et al., 1992). An examination of the maxicircle sequence reveals that the mRNA gene organization is very compact. Many of these genes overlap, and there is little room for regulatory sequences between the genes. Since mitochondrial transcription seems to employ polycistronic units and the formation of precise 5′ and 3′ ends often eliminates a portion of the coding sequence of the adjacent transcript, it is likely that these cleavage events regulate the abundance of the individual mRNAs, but virtually nothing is known about how this regulation might occur. Polyadenylation regulates gene expression in terms of mRNA stability and efficiency of translation in many systems including trypanosomes (Sachs and Wahle, 1993; Hotz et al., 1998) and may also have a role in the trypanosome mitochondrion. Indeed, the length of the poly[A] tails of some mitochondrial mRNAs in *T. brucei* is developmentally regulated in a transcript-dependent manner (Bhat et al., 1992; Read et al., 1992, 1994b). However, there is no direct evidence that cleavage or polyadenylation plays a role in mitochondrial expression. Clearly, the establishment of a trypanosome mitochondrial transcription–translation system is required to investigate the role of polyadenylation and processing in gene expression.

Since RNA editing creates translatable messages from nonsense sequences, it is perhaps the most appealing mechanism to regulate mitochondrial gene expression in

these cells. Nevertheless, there is as yet no conclusive proof that edited messages are translated in the trypanosome mitochondrion, and the role of RNA editing in regulating gene expression remains hypothetical. In *T. brucei*, pre-edited mRNAs are found almost exclusively with short poly[A] tails, whereas partially edited and fully edited mRNAs as well as transcripts that do not undergo editing may contain either short or long tails (Bhat et al., 1992; Militello and Read, 1999).This suggests that poly(A) tail length is not the sole signal for translation (Militello and Read, 1999). In addition, 3′ cleavage/polyadenylation and RNA editing seem to be independent events in *T. brucei* (Koslowsky and Yahampath, 1997). Thus, regulation of gene expression in the mitochondria of trypanosomes might be exerted at several levels, including processing of polycistronic transcripts into individual mRNAs, RNA editing, RNA stability and translation efficiency.

The steady-state abundance of edited transcripts is regulated in the life cycle of *T. brucei*. Mitochondrial function is repressed in the bloodstream form, and the abundance of some transcripts encoding components of the respiratory chain is severely diminished. One possible method to regulate editing is by regulating the levels of the gRNAs that edit developmentally regulated edited transcripts, but several studies have shown that there is no significant difference in the abundance of specific gRNAs of regulated genes throughout the life cycle of *T. brucei* (Koslowsky et al., 1992; Riley et al., 1995). Another possible mechanism to regulate editing is the differential expression of regulatory proteins that are preferentially expressed only in one stage of the life cycle. Alternatively, these proteins might be constitutively expressed, but they may undergo developmentally regulated posttranslational modifications such as phosphorylation. These regulatory proteins might be involved in stability of the edited RNAs (a possible candidate for this role might be mHEL61 described above) or might affect the editing efficiency of pre-edited mRNAs. Surprisingly, some mRNAs are constitutively edited in both life forms, and some edited transcripts are even more abundant in the bloodstream form, especially transcripts coding for components of Complex I of the respiratory chain (Koslowsky et al., 1990; Corell et al., 1994).

Although repressed, the mitochondrial function is not completely absent in bloodstream parasites, and there is even a cytochrome-independent electrochemical proton gradient across mitochondrial membranes that is probably created by the pumping of H^+ by the mitochondrial F_1F_0 ATPase (Nolan and Voorheis, 1992). A similar phenomenon is observed in another trypanosomatid, the plant parasite, *Phytomonas serpens*, that shows an RNA-editing pattern very similar to bloodstream *T. brucei* (Maslov et al., 1998, 1999). Interestingly, the gene coding for the ATPase subunit 6 is mitochondrial and undergoes editing in both procyclic and bloodstream *T. brucei* forms, as well as in *P. serpens* (Bhat et al., 1990; Maslov et al., 1999).

7. Conclusions and perspectives

Since the discovery of this phenomenon (Benne et al., 1986), U-insertion/deletion editing has been the focus of intensive research to elucidate its mechanism and significance. The number of Us inserted or removed from each mRNA is known precisely, and many but not all the gRNAs have been identified. Much insight has been gained by studying the editing reaction in vitro, especially in terms of distinguishing between the enzyme cascade and transesterification models of editing. Unfortunately, the in vitro assays available are only able to edit one editing site, and this inefficiently. Clearly, an assay that reproduces what occurs in vivo is needed in order to understand the editing reaction as a whole. With the possible exception of mHEL61p, no protein has been shown to be unambiguously involved in editing. The availability of recombinant editing proteins and specific antisera would be of great assistance in the isolation and characterization of the various editing complexes. Gene disruption experiments should also aid in the characterization and the role of the individual components. The use of bloodstream *T. brucei* cells in the gene knockout experiments should be especially valuable since editing occurs but is dispensable due to the non-mitochondrial metabolism of this stage of the parasite. In addition, the availability of a tetracycline-inducible expression system for conditional knockouts in bloodstream *T. brucei* (Wirtz and Clayton, 1995; Estévez et al., 1999; Wirtz et al., 1999) should also be helpful in this regard. Immunoprecipitation, immunodepletion and gene disruption data should be analyzed together to gain an insight into the function of gene products involved in RNA editing. An understanding of the mitochondrial transcription and translation systems may allow the development of mitochondrial selectable markers and a reverse genetic approach to studying editing in kinetoplastids. Clearly, a combination of biochemistry and genetics is essential for unraveling the editing reaction and its components and understanding the role of this peculiar phenomenon in the regulation of mitochondrial gene expression in these organisms.

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