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Composition of the editing complex of *Trypanosoma brucei*

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The RNA editing that produces most functional mRNAs in trypanosomes is catalysed by a multiprotein complex. This complex catalyses the endoribonucleolytic cleavage, uridylate addition and removal, and RNA ligation steps of the editing process. Enzymatic and *in vitro* editing analyses reveal that each catalytic step contributes to the specificity of the editing and, together with the interaction between gRNA and the mRNA, results in precisely edited mRNAs. Tandem mass spectrometric analysis was used to identify the genes for several components of biochemically purified editing complexes. Their identity and presence in the editing complex were confirmed using immunochemical analyses utilizing mAbs specific to the editing complex components. The genes for two RNA ligases were identified. Genetic studies show that some, but not all, of the components of the complex are essential for editing. The TbMP52 RNA ligase is essential for editing while the TbMP48 RNA ligase is not. Editing was found to be essential in bloodstream form trypanosomes. This is surprising because mutants devoid of genes encoding RNAs that become edited survive as bloodstream forms but encouraging since editing complex components may be targets for chemotherapy.

Keywords: RNA editing; RNA interference; terminal respiration; mass spectrometry; gene function; trypanosomes

1. INTRODUCTION

Most mitochondrial mRNAs in trypanosomes undergo RNA editing, a post-transcriptional maturation that inserts and deletes Us (Stuart *et al.* 1997, 2000; Estévez & Simpson 1999). This process occurs in all Trypanosomatids (e.g. *Trypanosoma*, *Leishmania*, and *Crithidia* species) although which mRNAs are edited, and the extent to which they are edited, differs among the species (Stuart 1991). The editing can be extensive with hundreds of Us inserted and tens of Us deleted in a single mRNA. Nevertheless, the final sequences are precise and predict proteins that are homologous to proteins encoded in the mitochondrial DNA of a wide range of organisms that do not edit their mitochondrial RNAs. The edited mRNAs encode components of the mitochondrial oxidative phosphorylation system (including subunits of complexes I (NADH dehydrogenase), III (Cyb, CYc), IV (cytochrome oxidase), and V (ATP synthase)), a mitochondrial ribosomal protein, and a protein with unknown function (MURF2). Amino-acid sequence analysis of CYb protein confirmed that the edited mRNAs are translated and are thus functional mRNAs (Horváth *et al.* 2000). Hence, production of a functional cytochrome-mediated oxidative phosphorylation system in trypanosomes requires editing of the mRNAs.

Editing of the mitochondrial mRNAs is regulated during the life cycle of trypanosomes and the pattern of which mRNAs are edited parallels the differences in energy metabolism between the life-cycle stages (Feagin *et al.* 1986). The mRNAs for cytochromes are edited in PFs of *Trypanosoma brucei* in which energy is generated by cytochrome-mediated oxidative phosphorylation but are not edited in slender BFs where energy is generated by glycolysis. The mRNAs for components of NADH dehydrogenase exhibit a reciprocal pattern of editing during the life cycle, being preferentially edited in BF. For example, the 5′ domain of ND7 mRNA is edited both in BF and PF but the 3′ domain is essentially only edited in BF. This suggests that the regulation of editing contributes to the alternation between terminal respiratory systems during the life cycle of trypanosomes.

The edited sequence is specified by small (*ca.* 60 nucleotide) gRNAs, each of which specifies the editing of a *ca.* 35 nucleotide block of mRNA sequence that contains about 10 ESs. Each gRNA has three sequence domains. The 5′ domain forms an anchor duplex with the mRNA directly 3′ to the block of mRNA sequence where editing will be specified by the gRNA. The central domain of the gRNA specifies the block of edited sequence. The 3′-end of each gRNA has an oligo (U) tail that is added post-transcriptionally (Blum & Simpson 1990). The function of this oligo (U) tail is unknown but it may stabilize the interaction between the gRNA and the mRNA, possibly when the mRNA block is almost completely edited. Editing of the mRNA proceeds from 3′ to 5′, with each gRNA utilized as it is able to form an anchor duplex with the

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mRNA. The gRNAs for the 3′ blocks of the editing domains form an anchor duplex with unedited mRNAs. Editing with these gRNAs produces the sequences that form anchor duplexes with gRNAs for the next blocks, and this process is repeated for each gRNA. Thus, most mRNAs are edited at numerous sites by multiple gRNAs.

Editing occurs by a series of coordinated catalytic steps. This was shown using an *in vitro* system that contains synthetic mRNA and gRNA and mitochondrial extract and results in gRNA specified editing at a single site (Kable *et al.* 1996; Seiwert *et al.* 1996; Igo *et al.* 2000). The mRNA is cleaved at the ES by endonuclease and Us are either added or removed at the 3′ end of the 5′ cleavage fragment depending on the interaction with the gRNA. The Us are added by 3′ TUTase or are removed by 3′ exoUase. The processed 5′ fragments are then rejoined by RNA ligase. As described below, the exquisite precision of editing is achieved by the substrate and catalytic specificities of the enzymes combined with the interactions between the gRNA and mRNA.

RNA editing is catalysed by a macromolecular complex, which has been referred to as the editosome. *In vitro* editing activity sediments at 20S (Pollard *et al.* 1992; Corell *et al.* 1996) and the composition of the complex is beginning to be revealed as described below, but its structure is not yet known. It has yet to be determined if it is a unitary catalytic complex, or has a stable catalytic core with which accessory factors dynamically interact, or consists of multi-protein subunits that dynamically interact during editing. Several candidate components of the editosome have been identified. Biochemical enrichment of editing complexes by various means resulted in fractions, which contain as few as 7 (Rusché *et al.* 1997), *ca.* 20 (Panigrahi *et al.* 2001), or 13 (Madison-Antenucci *et al.* 1998) major proteins. These divergent results may reflect the different purification and activity monitoring procedures, and consequential differential dissociation, of the complex and associated proteins. The candidate proteins have been studied further, as described below. In addition, Göringer and colleagues (Missel *et al.* 1997), having deduced that editing would require an RNA helicase, cloned a mitochondrial RNA helicase, mHel61, and showed that null mutant PFs have diminished editing. Thus, mHel61 may have a non-essential role in editing. gBP21 (Köller *et al.* 1997), RBP16 (Hayman & Read 1999), and TbRGG1 (Vanhamme *et al.* 1998) are mitochondrial RNA binding proteins and REAP1 (Madison-Antenucci *et al.* 1998) co-sediments with editing complexes. The presence, or association with, the editing complex, or a role in editing has not yet been confirmed for these proteins. On the contrary, two candidate editing ligases were recognized by their autoadenylation (Sabatini & Hajduk 1995) and have been shown to be components of the editing complex (McManus *et al.* 2001; Rusché *et al.* 2001; Schnaufer *et al.* 2001) and one ligase is essential for editing (Schnaufer *et al.* 2001). We report here the characteristics of editing catalytic activities that contribute to the precision of RNA editing, the identification of multiple proteins and corresponding genes for components of the editing complex, and the roles of some of these components in RNA editing.

2. ACTIVITIES ASSOCIATED WITH THE EDITING COMPLEX

The editing complex contains the endonuclease, exonuclease, TUTase, and RNA ligase activities that catalyse the steps of editing and act in concert along with the gRNA to specify the precise edited mRNA sequence. Endonuclease activity was monitored during purification of the editing complex (described below) by assaying gRNA-directed cleavage of CYb mRNA. The gRNA-directed cleavage activity co-fractionated with *in vitro* editing through two ion-exchange columns, a gel filtration column and glycerol gradient sedimentation. An endonuclease activity that cleaved CYb at another site in the absence of gRNA separated from this activity on the first ion-exchange column. RNase P activity, which was monitored using a pre-tRNA substrate, co-fractionated with *in vitro* editing through the two ion-exchange columns but fractionated away from *in vitro* editing during gel filtration chromatography. *In vitro* analyses, with a variety of substrates, revealed that the editing endonuclease normally cleaves mRNA at the site immediately 5′ to a continuous anchor duplex, requires the Sp phosphate isomer, and leaves the phosphate on the 3′ cleavage product. Some nucleotide substitutions immediately flanking the ES resulted in a blockage of cleavage or shifting to other sites, and a bias against cleavage 3′ to C nucleotides was observed (figure 1*a*). Thus, we conclude that the editing endonuclease activity normally cleaves mRNA immediately 5′ to a continuous (anchor) RNA duplex but that specificity is limited, perhaps reflecting the RNA structure, interaction of RNA with the editing complex, and/or multiple endonucleases in the editing complex. This specificity is consistent with the existence of partially edited sequences at the junction of edited and unedited regions in partially edited mRNAs.

The peak of exonuclease activity from the first ion exchange column (SP Sepharose) was broad, suggesting the presence of other exonuclease activities, and was especially evident using the single-stranded substrate. The editing exonuclease was found to be specific for removal of Us which are not base paired with the gRNA. This was found with both the original *in vitro* editing assay, which requires cleavage by the endogenous editing endonuclease (Seiwert *et al.* 1996), and the pre-cleaved assay in which the mRNA is provided as two 'pre-cleaved' fragments (Igo *et al.* 2000). Assays using substrates with non-U nucleotides substituted within oligo (U) sequences that are normally removed resulted in U removal up to the substituted nucleotide (figure 1*b*). In addition, the use of gRNA that could base pair with Us that were normally removed prevented their removal. This specificity for removal of Us that are not base paired with the gRNA is consistent with the model of the editing mechanism and will contribute to the editing precision.

The mitochondrial extracts contain substantial 3′ terminal U addition activity, which adds to both single- and dsRNA (N. Ernst and K. Stuart, unpublished data). Several peaks of activity eluted from the first ion-exchange column (SP Sepharose) and the gel filtration column. Only the activity that co-eluted with the editing activity was characterized further. The TUTase activity that co-purified with the 1600 kDa complex that catalyses the four

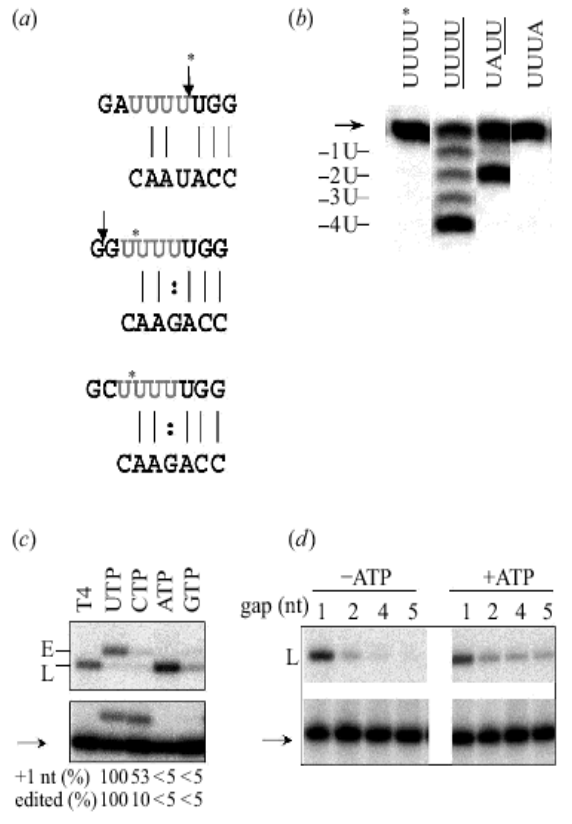


Figure 1. Specificity of enzymatic activities in RNA editing. (*a*) ES sequences showing where cleavage is expected 5′ to the anchor duplex (asterisk) and where cleavage actually occurs (arrow). Vertical lines indicate Watson–Crick base pairs and the colons represent G : U base pairs. (*b*) Pre-cleaved deletion editing assay showing removal of Us only 3′ to an A that is substituted for a U that is normally removed by editing. The 5′ labelled input 5′ RNA has four 3′ terminal Us (arrow), which are not removed in the absence of extract (asterisk), but are normally removed (second lane) as indicated by underlining. Us 3′ to the substitute A are removed (third lane) and a 3′ terminal A blocks any U removal. (*c*) Addition (lower panel) and ligation (upper panel) products of pre-cleaved editing reactions using a gRNA that specifies insertion of one nucleotide. U was added at approximately double the level of C (including that becoming edited—E) and almost no A or G was added using 100 μM NTP. Edited RNA (E) preferentially contained the added U and ligation of products without addition (L) was enhanced by addition of ATP. The two fragments ligated using T4 ligase were used as a size standard. (*d*) Specificity of ligation. Ligation of substrates with gaps of one to five nucleotides were assayed in the presence and absence of 0.3 mM ATP. The input (arrow) and ligated (L) RNAs are indicated.

steps of editing (see below) was specific for the addition of Us. An indistinguishable activity co-purified with a 500 kDa complex that catalysed some steps of the editing process (see below) but a second activity that added numerous Us was also present in this fraction. The presence of multiple TUTase activities is not surprising since Us are not only added within edited mRNAs but also to the 3′ ends of gRNAs, to the 3′ ends of mitochondrial rRNAs, and within mRNA poly(A) tails. Further work is needed to determine if there are different TUTase proteins or if there is a single TUTase protein that differentially associates with other proteins. The TUTase activity that co-purifies with the editing complex adds Us (from UTP) in preference to Cs, and essentially does not add A or G (figure 1*c*). A single U is efficiently added regardless of the opposing nucleotide in gRNA. However, addition of more than one U proceeds up to, but not beyond, the number specified by the gRNA. Furthermore, molecules with an added C or a number of Us not specified by the gRNA are selectively excluded from the edited RNA as described below. Thus, the specificity of TUTase for U as influenced by the interaction with gRNA contributes to the precision of editing.

RNA ligase activity, which was monitored by a substrate RNA ligation assay and by adenylation of two proteins, co-purified with *in vitro* editing (S. Palazzo and K. Stuart, unpublished data). The ligase activity that co-purified with the editing complex requires a bridging RNA or DNA, ligates RNA with DNA, but does not ligate two DNAs. Its ligation was most efficient with RNAs that lack a gap (i.e. RNAs with terminal nucleotides base paired with adjacent nucleotides of the bridging RNA), unlike T4 RNA ligase which prefers a gap of two nucleotides. Ligation efficiency diminished in proportion to gap size (figure 1*d*). Similarly, ligation was most efficient with no overhanging 3′ nucleotides, although 3′ exonuclease activity in the complex was simultaneously active. The elimination of base pairs by C and/or U substitutions for guiding As or Gs in the gRNA substantially reduced ligation and resulted in misedited RNA in some cases as an apparent result of alternative base pairing between the mRNA and gRNA. Base pairing between gRNA and the mRNA nucleotides that flank the ligation site enhanced ligation. Ligation by the purified complex occurs without added ATP due to the ligases being pre-adenylated. Deadenylation by incubation with 40 $\mu g\,ml^{-1}$ ligatable yeast RNA completely abolished ligation activity and most activity was restored (after RNA removal) by the addition of 0.3 mM ATP. Similarly, ligation was inhibited by the addition of 4 mM PPi and restored by the addition of ATP or removal of PPi. The addition of ATP to purified editing complexes increased editing but reduced accuracy, perhaps due to an increase in the rate of ligation over U addition or deletion. Thus, preferential ligation of 5′ fragments with the number of Us specified by the gRNA contributes to the accuracy of editing.

The TUTase and ligase steps each contribute to the accuracy of editing and their activities may be coordinated. In the absence of ligation, U addition activity is diminished, but its accuracy (i.e. addition of the number specified by gRNA) is retained when ligation is blocked with PPi. U addition is also diminished when ligation is blocked by the use of a 3′ fragment lacking a 5′ phosphate. In the absence of U addition by omission of UTP, RNA ligation is efficient with no added ATP and unaffected by the addition of up to 20 mM ATP when there is no gap. The presence of a gap dramatically diminishes ligation without added ATP and addition of ATP promotes ligation while accuracy is diminished.

Overall, each enzyme contributes some specificity to the editing process, and together with the specificity conferred by base pairing with the gRNA, accounts for the accuracy with which the edited sequence is determined.

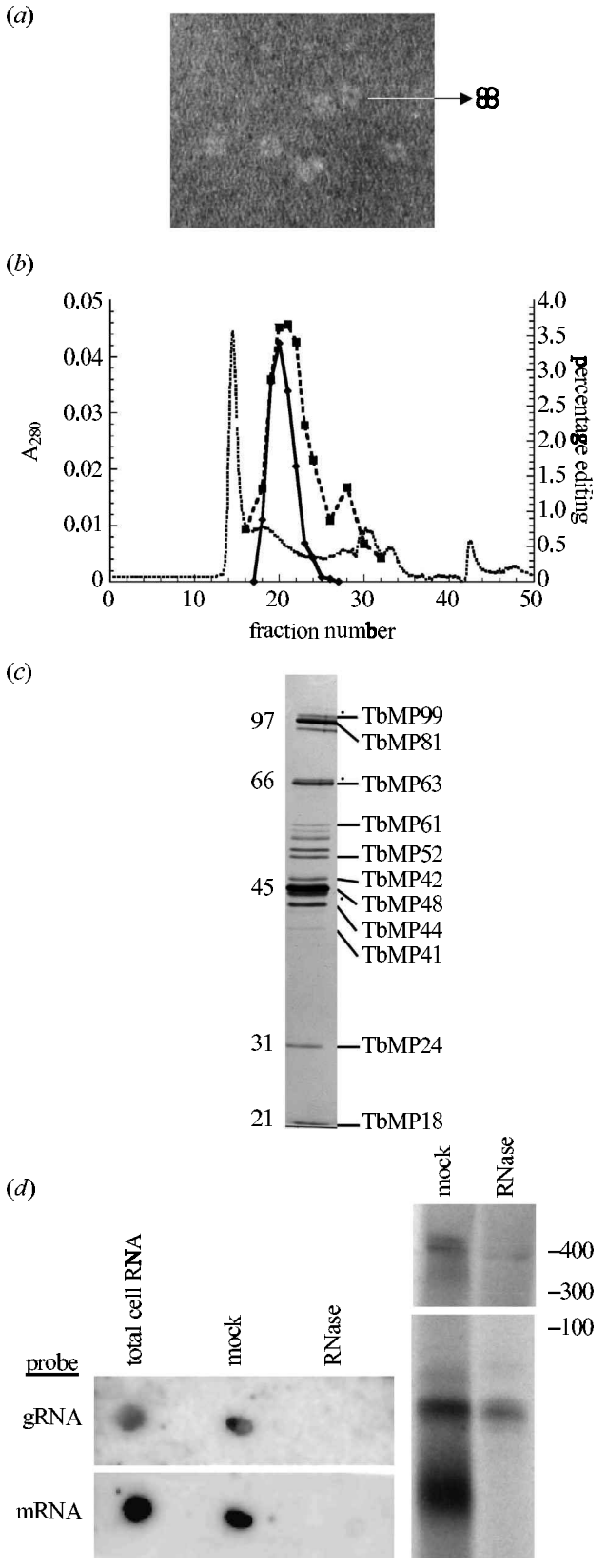


Figure 2. (*a*) Transmission electron micrograph showing the tetrameric structure of purified 20S complexes. (*b*) Elution profile of the RNA editing complex from a Superose 6 column following two ion-exchange columns. Deletion editing (diamonds) and pre-cleaved editing (squares), are expressed as the percentage of edited substrate ×2 and the dotted line indicates absorbance at 280 nm. (*c*) Silver-stained SDS–PAGE gel of purified 20S complexes. Candidate complex proteins identified by MS–MS analyses are indicated and contaminants GDH, hsp70, and ATP synthase-α are indicated by a dot. (*d*) RNA in immunoaffinity-purified editing complexes. Dot–blot hybridization with oligonucleotide probes showing loss of both gRNA and mRNA by digestion with RNAse but not by mock digestion (left panel). Post-labelled RNA in mock and RNAse-digested editing complexes separated on 9% denaturing polyacrylamide gel (right panel).

3. THE EDITING COMPLEX

The editing complex must contain multiple proteins to perform the catalytic steps of editing, bind RNA, translocate RNA during this process, and maintain structural integrity. It may contain structural or catalytic RNA (i.e. not a gRNA or mRNA) as do ribosomes and spliceosomes. The editing complex was biochemically purified in order to identify its components and characterize their functions. Purification was monitored with two *in vitro* editing assays and the independent catalytic activities were also assayed. The traditional deletion editing assay (Seiwert *et al.* 1996) requires concerted endonuclease, exonuclease and RNA ligase activities of editing (Kable *et al.* 1997), and hence a full round of editing. The pre-cleaved insertion assay provides the mRNA as two fragments and requires TUTase and RNA ligase but not endonuclease activities. The *in vitro* deletion editing activity that was enriched from mitochondrial lysates by sequential cation-exchange and anion-exchange columns, eluted with an apparent mass of *ca.* 1600 kDa from gel filtration columns, and sedimented at *ca.* 20S in glycerol gradients (Panigrahi *et al.* 2001). Insertion editing sedimented at a somewhat higher S value. Transmission electron microscopy of the purified 20S fraction revealed a tetrameric structure (figure 2*a*). Nuclease, TUTase, and RNA ligase activities co-purified with the complex that catalysed *in vitro* deletion editing. However, in general these activities were broadly distributed in the cation-exchange column. This may reflect the interaction between different components of the complex and the ion exchanger, fragmentation of the complex during purification, and/or the presence of catalytic activities that are unrelated to editing. A second smaller peak of these activities, and of pre-cleaved editing activity, eluted from the gel filtration column at *ca.* 500 kDa (figure 2*b*). These activities may be related to the *ca.* 700 and *ca.* 450 kDa complexes with adenylatable proteins that were observed by the Hajduk laboratory using a different purification procedure (Madison-Antenucci *et al.* 1998). The 1600 kDa complex catalyses all four steps of editing while the *ca.* 500 kDa complex lacks endonuclease activity and some proteins present in the 1600 kDa complex, as described below. Hence, the smaller complexes may represent subunits or fragments of editing complexes.

Fifteen out of the 20 proteins that were observed by SDS–PAGE analysis of the editing complexes from the final glycerol gradient step were identified, along with their corresponding genes, by MS–MS (figure 2*c* and table 1). Identification of the other five proteins awaits completion of the *T. brucei* genome sequence. Both the total fraction and individual gel bands were digested with trypsin, fractionated by capillary liquid chromatography, and directly eluted into the mass spectrometer (Gygi *et al.*

Table 1. Candidate editosome proteins.
(Abbreviations: IP–W, in immunoprecipitate (IP) by Western analysis (W); IP–M, in IP by tandem mass spectrometry; IP–A, in IP by autoadenylation (A); GG–W, co-sediments with complex in glycerol gradient by W; GG–A, co-sediments with complex by A; ND, no data.)

identity	motif	in complex	effect on editing	potential role
TbMP99	—	IP–M	ND	—
TbMP81	Zn finger	IP–W, IP–M, GG–W	RNAi blocks editing	endonuclease molecular interaction
TbMP63	Zn finger	IP–W, IP–M, GG-W[a]	ND	molecular interaction
TbMP61	DEAD box	—	reduced editing in null mutant	RNA helicase
TbMP52	ligase	IP-W, IP-M, GG-A, IP-A[a]	inactivation blocks editing	RNA ligase
TbMP48	ligase	GG-A, IP-A, IP-M	inactivation does not block editing	RNA ligase
TbMP44	RNAse III-like	IP-M	inactivation blocks editing	nuclease
TbMP42	Zn finger	IP-W, IP-M, GG-W	ND	molecular interaction
TbMP24	Arg rich	IP-M	ND	—
TbMP18	—	IP-M	ND	—

[a] TbMP63 and TbMP52 interact *in vitro*.

1999; Panigrahi *et al.* 2001). The resultant CID spectra of each peptide were compared to spectra predicted from all six ORFs from the *T. brucei* sequence database using SEQUEST software to identify the corresponding genes. Multiple peptide matches were found for genes for TbMP52 (Panigrahi *et al.* 2001), TbMP48, TbMP81, TbMP61 (mHel61), glutamate dehydrogenase, and hsp70, which had complete sequences in the *T. brucei* sequence database. Nine other proteins were identified from analyses of partial sequences in the *T. brucei* and other trypanosome databases (table 1). Because their complete ORFs were not available in the databases, the corresponding genes were identified by iterative BLAST analyses of genomic or EST sequences with significant matches to the CID spectra to identify overlapping sequences followed by PCR amplification, cloning, and sequencing of the complete ORF, and analysis of the CID spectra to confirm gene identity. Three proteins, glutamate dehydrogenase, hsp70, and ATP synthase-α were found to be contaminants, perhaps due to their abundance, and/or affinity for RNA or protein. Western analysis with mAbs specific for these proteins showed that they did not co-sediment in glycerol gradients with the editing complex, and peptides corresponding to these proteins were not found by MS–MS analysis of immunoprecipitates using mAbs specific for editing complex proteins.

At least 10 proteins are apparent components of the editing complex. Four mAbs from a panel that was generated using the glycerol gradient fraction (Panigrahi *et al.* 2001) reacted with native and recombinant TbMP81, TbMP63, TbMP52, and TbMP42, thus confirming the identity of these genes. These mAbs immunoprecipitate *in vitro* editing from the 20S fraction and from total cell lysates. The anti-TbMP81 immunoprecipitate catalyses pre-cleaved editing but not deletion editing, the significance of which is discussed below. Each immunoprecipitate contains all four proteins as determined by Western and MS–MS analyses. All four proteins co-fractionate in glycerol gradients and by gel filtration as shown by Western analysis, thus indicating that they are in the same complex. TbMP42 was not detected in the 500 kDa complexes by Western analysis suggesting that this complex is a subunit or fragment of the editing complex. Peptides corresponding to TbMP18, TbMP24, TbMP44, TbMP48, and TbMP99 were identified by MS–MS analysis of the mAb immunoprecipitates (table 1) indicating that they are also present in the complex. TbMP61 was identified as the mHel61 RNA helicase that has a possible role in editing (Missel *et al.* 1997). However, it was not detected in immunoprecipitates and thus may be loosely associated with the complex. Purified and immunprecipitated editing complexes contain endogenous gRNA and mRNA and *ca.* 60 nucleotide RNA remains after RNase treatment (figure 2*d*). It is unclear if this residual RNA is protected gRNA and/or mRNA or structural or catalytic RNA.

TbMP41 and TbMP90 were not detected in immunoprecipitates and thus their presence in the complex is uncertain. Similarly, the gRNA binding protein gBP21, oligo (U) binding proteins RBP16, TBRGG1, and REAP1 which co-sediment with editing complexes, were not detected in biochemically purified or immunoprecipitated editing complexes either by MS–MS or Western analyses. Hence, these proteins do not appear to be stable components of the editing complex. Thus, the proteins in the 1600 kDa complex may be components of a stable catalytic core complex. The other proteins may have a role in editing that is indirect or entails transient association with the catalytic core complex, or they may have no role in editing. The 500 kDa and the 750 and 450 kDa complexes observed by others (Madison-Antenucci *et al.* 1998) may be subunits or fragments of the editing complex and may have arisen as a result of fractionation. The smaller complex purified by others (Rusché *et al.* 1997) possibly reflects a procedure that removed RNA and hence some protein components of the complex.

4. FUNCTIONS OF EDITING COMPLEX COMPONENTS

The editing complex must contain components that catalyse the steps of editing, bind the mRNA and gRNA, and position them for catalysis. It must also contain components that translocate the RNAs so that each of the

several sites specified by a gRNA are edited. They must also function in maintaining a complex structure and integrity and in the regulation of editing during the life cycle.

(a) *Editing ligases*

TbMP48 and TbMP52 were found to contain ligase motifs and to be related to each other (figure 3*a*). They have 41% and 60% amino-acid sequence identity and similarity, respectively, and we identified *Leishmania major* homologues for these genes. The sizes of the predicted proteins corresponded to two adenylatable proteins that had been identified as editing ligases. Recombinant TbMP48 and TbMP52 autoadenylate, the adenylated proteins have the same mobility as the smaller and larger adenylated native proteins, respectively (McManus *et al.* 2001; Schnaufer *et al.* 2001). A mAb specific for TbMP52 immunoprecipitates the larger native adenylated protein after dissociation of the complex with SDS and dilution. Recombinant TbMP48 and TbMP52 that were immunoprecipitated with anti-his tag or anti-TbMP52 mAb, respectively, both catalysed ligation of synthetic RNA. Thus, TbMP48 and TbMP52 are RNA ligases that correspond to the smaller and larger adenylatable proteins present in purified editing complexes.

Regulatable TbMP48 sequences to produce interfering dsRNAi were introduced into both the bloodstream and procyclic stage cell lines, as described (Shi *et al.* 2000; Wang *et al.* 2000). We were unable to produce null mutants of TbMP52 and thus produced mutant BFs in which we introduced a regulatable TbMP52 allele and then deleted both endogenous alleles in BFs as described by Wirtz *et al.* (1999). Inactivation of TbMP48 and TbMP52 gene expression was confirmed by Northern and Western analyses, respectively. The addition of tetracycline resulted in the loss of TbMP48 RNA that could be detected by Northern analysis and removal of tetracyline resulted in substantial reduction in TbMP52 protein. The loss of TbMP48 did not affect editing or cell growth in either life-cycle stage of the parasite. Thus, the TbMP48 ligase is not essential for editing, possibly because the TbMP52 ligase may be able to compensate for its loss. In contrast, the reduction in TbMP52 expression resulted in cessation of RNA editing (figure 3*b*) followed by cell death (figure 3*c*). Fully edited A6, ND7, and RPS 12 RNA were not detectable by RT-PCR after two days of gene inactivation following removal of tetracycline while the level of edited mRNAs was indistinguishable from that in wild-type cells when the TbMP52 gene was active. The spectrum of partially edited molecules diminished both in size and abundance until they were almost undetectable after three days. More quantitative poison primer extension analysis showed more than a 50-fold reduction of the edited ND7 mRNA within 75 h of tetracycline removal (figure 3*b*). Thus, TbMP52 is essential for editing and TbMP48 cannot compensate for its absence.

Normal cell growth continued for 48 h after the loss of fully edited RNA upon TbMP52 inactivation, presumably reflecting the turnover rates of the proteins encoded by the edited mRNAs. Reactivation of TbMP52 gene expression by the reintroduction of tetracycline following cessation of growth resulted in a resumption of cell growth after a three day lag (figure 3*c*). Mice infected with the *T. brucei* strain containing the regulatable TbMP52 gene died unless tetracycline was added to the drinking water, showing that inactivation of the gene *in vivo* was also lethal to the parasite. The lethality of loss of editing to the BF of *T. brucei* suggests that editing is normally essential to BFs and trypanosome strains and mutants that lack, or have severely abnormal, editing have undergone genetic or physiological compensation. It also raises the important possibility that the RNA editing process may provide chemotherapeutic targets.

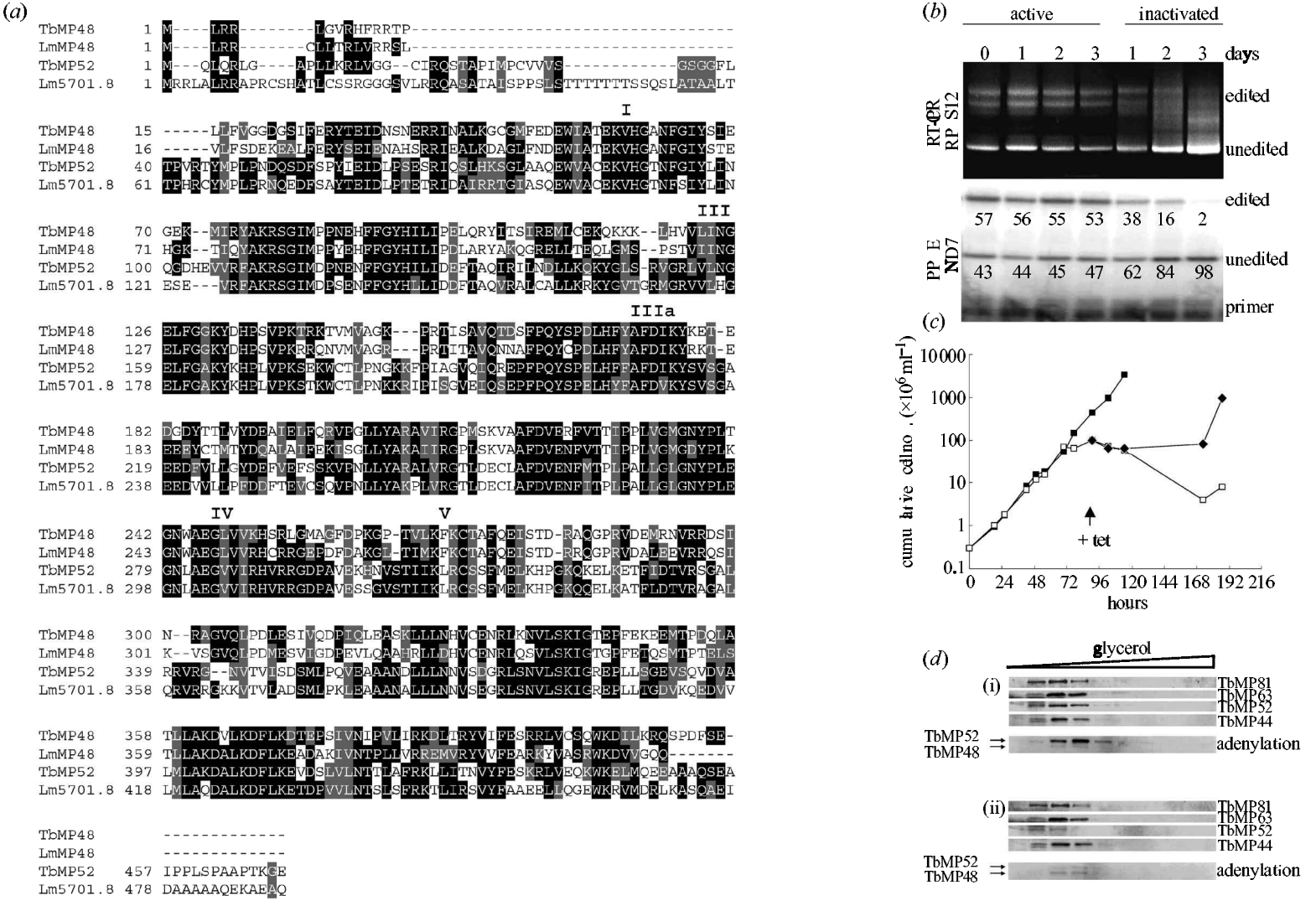
Editing complexes from cells with inactivated TbMP52 have a lower adenylatable TbMP52 : TbMP48 ratio compared to cells with active TbMP42. However, the complexes sediment at 20S (figure 3*d*) and their immunoprecipitated complexes catalyse *in vitro* pre-cleaved editing and ligation although at a reduced level, and with an altered specificity of ligation. Thus, editing complexes are present but with altered composition and activity. The presence of some residual TbMP52 and the potential tetrameric structure of the editing complexes obscure the detailed effects of TbMP52 loss to the editing complex structure and function.

(b) *Endonuclease*

A mAb specific for TbMP81 immunoprecipitated editing complexes that catalyse pre-cleaved editing (which does not require endonuclease activity), but not complete editing. This suggests that this antibody may block this catalytic activity, possibly by binding the catalyst. TbMP81 contains a single zinc finger motif but no other obvious motifs that would suggest its function. When expression of TbMP81 was inactivated by the regulated production of RNAi in both bloodstream and procyclic *T. brucei* there was a significant reduction in the production of edited RNA (as determined by RT-PCR analysis) and inhibition of growth after five days. Complexes that were immunoprecipitated from cells with downregulated TbMP81 using a mAb specific for TbMP63 had diminished endonucleolytic activity but ligation activity was unaffected. These data suggest that TbMP81 is associated with endoribonuclease activity and is essential for editing in and survival of both stages of the life cycle.

(c) *Protein relationships*

Four protein components of the editing complex, TbMP81, TbMP63, TbMP42, and TbMP18, had varying degrees of relationships to each other (figure 4). TbMP81 has a single C_2H_2 zinc finger while TbMP63 and TbMP42 each have two C_2H_2 zinc fingers. These zinc fingers may mediate RNA–protein or protein–protein interactions as has been suggested for spliceosomal proteins (Lygerou *et al.* 1999). Indeed recombinant TbMP63 and TbMP52 co-immunoprecipitate, suggesting a capability for direct interaction (A. Schnaufer, A. K. Panigrahi and K. Stuart, unpublished data). All four proteins share a conserved amino-acid sequence motif near their C-terminus and scattered amino acids throughout their sequences show that all are distantly related. In addition, TbMP18 has substantial homology to the C-terminal portion of TbMP42 but also has a short C-terminal extension. The presence of four related proteins, one of which (TbMP81) appears to be associated with endonuclease activity, suggests that some of the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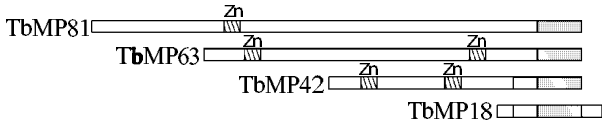

Figure 4. Diagram of the relationships between TbMP81, TbMP63, TbMP42, and TbMP18 proteins. A C-terminal sequence is conserved among the four proteins (shaded and cross-hatched) and a larger region of homology exists between TbMP42 and TbMP18 (shaded). The C_2H_2 zinc fingers are identified (Zn labelled and cross-hatched).

others may also have a related function, perhaps reminiscent of the TbMP48 and TbMP52 RNA ligases. It may also suggest the conservation of non-catalytic functions such as RNA and/or protein binding. Experiments assessing the effects of inactivation of TbMP18 with RNAi have shown inhibition of growth. This is also the case with TbMP24 and TbMP99. In addition, regulated inactivation of TbMP44 expression resulted in inhibition of growth and cessation of editing. Thus, TbMP18, TbMP24 and TbMP99 are candidate components of the editing complex while TbMP44 appears to be a component of the complex that is essential for editing.

(d) *Accessory factors*

It is probable that editing and, in particular, its regulation, entails the action of molecules that are not part of the catalytic core of the editing complex. Several candidates have been identified although their roles have not been established. Prominent in this category is the mHel61p RNA helicase. Insect form null mutants grow slowly and demonstrate a substantial reduction in edited mRNAs which is restored upon reintroduction of the mHel61 gene (Missel *et al.* 1997). Their mitochondrial extracts are capable of *in vitro* editing. These results, along with the immunoprecipitation studies outlined above, suggest that the RNA helicase can associate with the editing complex and may have a non-essential role in editing. However, helicase activity may be essential since there was no reduction in RNA-unwinding activity in the null mutant compared to wild-type implying the presence of one or more additional mitochondrial helicases. Similarly, null mutants of gBP21 grow and edit normally as BFs but cannot differentiate into insect forms (Lambert *et al.* 1999). Thus, gBP21 is not essential to editing in BFs but may have an essential role in insect forms and/or an indirect role, perhaps involving gRNA binding. Immunoprecipitation of editing complexes with mAbs specific for gBP21 show that it can associate with the editing complex although ablation of this immunoprecipitation by RNase treatment suggests that the association may be by RNA binding and hence perhaps does not represent a functional relationship to editing. We look forward to reading research reports on other genetic studies involving RBP16, TBRGG1, and REAP1.

5. PERSPECTIVE

The identification of the editing complex and some of its catalytic components does not identify the processes that led to the development of RNA editing nor the selective pressures that retained it. However, it shows that a complex process can arise from what appear to be conventional components. These components may have evolved into other processes as unanticipated as RNA editing or dsRNAi.

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