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# TbMP44 Is Essential for RNA Editing and Structural Integrity of the Editosome in *Trypanosoma brucei*

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**RNA editing produces mature mitochondrial mRNAs in trypanosomatids by the insertion and deletion of uridylates. It is catalyzed by a multiprotein complex, the editosome. We identified TbMP44 among the components of enriched editosomes by a combination of mass spectrometry and DNA sequence database analysis. Inactivation of an ectopic TbMP44 allele in cells in which the endogenous alleles were disrupted abolished RNA editing, inhibited cell growth, and was eventually lethal to bloodstream form trypanosomes. Loss of TbMP44 mRNA was followed initially by a reduction in the editosome sedimentation coefficient and then by the absence of other editosome proteins despite the presence of the mRNA. Reactivation of *TbMP44* gene expression resulted in the resumption of cell growth and the reappearance of editosomes. These data indicate that TbMP44 is a component of the editosome that is essential for editing and critical for the structural integrity of the editosome.**

RNA editing in the mitochondria of kinetoplastid protozoa is a form of RNA processing that occurs by a series of enzymatic steps and results in mature mRNAs (9,18,34,35). During this process, mitochondrial (mt) pre-mRNA is cleaved by endonuclease at a site specified by guide RNA (gRNA). Uridylates (U's) are then either removed from or added to the 3′ end of the resultant 5′ mRNA fragment by a uridylate-specific 3′ exoribonuclease (exoUase) or 3′ terminal uridylyl transferase (TUTase), respectively. The processed 5′ fragment is then rejoined to the 3′ fragment by RNA ligase to complete one catalytic cycle of editing. Each gRNA specifies the editing of multiple sites, thus employing multiple catalytic cycles to edit a block of sequence. Most mRNAs undergo extensive editing in *Trypanosoma brucei,* during which several gRNAs are used, each specifying the editing of one of the multiple blocks of edited sequence, resulting in hundreds of U insertions and tens of U deletions. The edited RNAs encode components of the oxidative phosphorylation system including subunits of the NADH ubiquinone, cytochrome *b*/*c,* cytochrome oxidase, and ATP synthase complexes. Cytochrome-mediated oxidative phosphorylation is active and essential in procyclic forms of the insect stage of the life cycle. However, components of this system, including cytochrome *b* and cytochrome oxidase subunit 2, are not present in the bloodstream form in the mammalian stage of the life cycle (28). Nevertheless, inactivation of editing is lethal to bloodstream forms (33).

RNA editing is catalyzed by a multiprotein complex, the editosome. Complexes that catalyze in vitro editing and thus must contain the proteins responsible for the endoribonuclease, TUTase, exoUase, and RNA ligase activities sediment in glycerol gradients at ~20S (26). Biochemical purification of these complexes and activities from procyclic *T. brucei* mitochondria by various methods resulted in preparations that contained 7 to more than 20 major protein bands (19, 26, 29) and particles with four protuberances (27, 36). The presence of several of these proteins in editosomes has been confirmed, and the functions of some proteins in editing have been identified (7, 13, 21, 30, 33). In addition, several mitochondrial proteins with potential roles in editing have been identified primarily based on their RNA binding activity. However, they do not appear to be contained in the 20S editosome and thus may be accessory factors (34). These potential accessory factors include gBP21 (16) and the related gBP25 (4), as well as REAP-1 (19), RBP16 (11), and TbRGG1 (38). gBP21 does not appear to be essential since null mutants of gBP21 retain editing (16, 17). However, a role in editing for gBP21 is not excluded, possibly due to the presence of a related protein, gBP25 (4). While some protein components of the editosome have catalytic activities, others enhance the activities of the catalysts, play roles in RNA binding and translocation, function in accessory protein interactions, or maintain editosome structural integrity.

We identified 21 potential editosome proteins and their corresponding genes in this laboratory by using various editosome purification techniques combined with mass spectrometry (34). Two of these are RNA ligases (33) that were first visualized by adenylation (31), shown to be in preparations of purified complexes, and designated bands V and IV (30). The genes for these proteins were identified and shown to have ligase motifs and to encode proteins with RNA ligase activity (33). These were initially designated TbMP48 (band V) and TbMP52 (band IV) since they encode 48- and 52-kDa preprocessed proteins and subsequently TbREL2 and TbREL1 for RNA editing ligases 2 and 1 (21, 29, 30, 33). Inactivation of TbREL1 gene expression in transgenic *T. brucei* blocks editing, showing that it is essential for this process (33). Inactivation of TbREL2 gene expression does not block editing (7), but this may reflect

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the ability of TbREL1 to compensate for its loss in a nonreciprocal fashion, since these two proteins have considerable sequence similarity. Five other editosome proteins, TbMP18, TbMP24, TbMP42, TbMP63, and TbMP81, of which the largest three have zinc finger motifs, have a complex pattern of sequence relationships (27; R. Salavati et al., unpublished data). TbMP18, TbMP42, TbMP63, and TbMP81 correspond to bands VII, VI, III, and II as designated by the Sollner-Webb laboratory (29). Studies to date (7, 13), as well as those still in progress, indicate that these proteins are essential for editing and show specific interactions with the other components of the editosome as detailed in the Discussion. Importantly, inactivation of TbMP81 gene expression (7) results in preferential loss of TbREL2 and insertion editing while inactivation of TbMP63 (band III) results in loss of TbREL1 (13). This, along with different enzymatic characteristics of the ligase activities, suggests that insertion and deletion editing are functionally and physically separate.

Of the other 14 potential editosome proteins that we identified in purified editosomes, 9 are clearly components of the complex based on a combination of studies that identified or inferred their functions, effect on editing, and association with or relationship to other editosome proteins (25a). The other five proteins are less well studied. One protein, mHel61, encodes an RNA helicase, and its inactivation diminishes editing (24). The incomplete loss of editing shows that mHel61 is not essential for editing and that another helicase, yet to be identified, may also function in editing. A 57-kDa editosome protein has TUTase activity but its requirement in editing is not yet known (34; N. Ernst et al., submitted for publication), while a 108-kDa TUTase is essential for editing (2) but has not been detected in the editosome (25a). Two related editosome proteins, TbMP99 and TbMP100, have endo/exonuclease motifs, while three other related editosome proteins contain RNase III or RNase III-like motifs and are related to two additional related proteins (25a, 34) including TbMP44, which is the focus of this study.

We report here that TbMP44 is an essential component of the editosome since inactivation of *TbMP44* gene expression in vivo results in the loss of editing. The inactivation is lethal in bloodstream forms, further indicating that editing is normally essential in this stage of the life cycle. Inactivation is initially followed by structural changes and then loss of editosomes, indicating that TbMP44 plays a role in editosome structural integrity. Reactivation of the *TbMP44* gene results in the reappearance of editosomes and the return of editing.

## MATERIALS AND METHODS

**Cell growth and preparation and analysis of nucleic acids.** *T. brucei* bloodstream forms (strain 427) were grown in HMI-9 medium at 37°C and transferred every 48 h to maintain the log phase growth (12). Genomic DNA was extracted from bloodstream forms by a method adapted from that of Bellofatto and Cross (3). Total cellular RNA was prepared using Trizol as specified by the manufacturer (GIBCO-BRL), and contaminating DNA was removed by treatment with RQ1 RNase-free DNase (Promega) at 37°C for 15 min. RNA was quantified by UV spectroscopy. For Northern analysis, 20 μg of total RNA was electrophoresed on 1% agarose–formaldehyde gels, transferred to Hybond Plus membrane (Amersham), and hybridized to radiolabeled riboprobes in Rapid-Hyb buffer (Amersham), as specified by the manufacturer, with 0.16- to 1.77- and 0.24- to 9.5-b RNA ladders (GIBCO-BRL) serving as size markers. The riboprobe was in vitro transcribed from the PCR product amplified from genomic DNA of *T. brucei* (strain 427) with primers 3324 (5′-ATGAGACGGGCTGTGGTAC-3′) and 3977 (5′-TGTAATACGACTCACTATAGGGCCGCCCTCCCAGTGCCAG-3′) (the T7 promoter is underlined). For Southern analysis, 5 μg of genomic DNA was resolved on a 1% agarose gel. A PCR product from genomic DNA was labeled using the High Prime kit (Roche) as specified by the manufacturer and used to probe the blot as described for the Northern hybridization.

**Identification of the *TbMP44* gene and protein.** *T. brucei* editosomes were isolated from mitochondria by sequential ion-exchange and gel filtration column chromatography followed by glycerol gradient sedimentation and by immunoprecipitation using monoclonal antibody (MAb) P1H3, which is specific for the TbMP63 editosome protein, as described previously (25a, 26, 27). The TbMP44 protein and corresponding gene were identified by mass spectrometric analysis of purified editosomes and of a protein band cut from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and comparison to The Institute for Genomic Research (TIGR) *T. brucei* 927 DNA sequence database (www.tigr.org) as described previously (27). A portion of the open reading frame (ORF) was initially identified in genomic clone 24M16 (provided by TIGR). A PCR product made based on this partial ORF sequence was used as a probe to identify *T. brucei* 927 P1 genomic DNA clone M1H4 on filter SM12#61 (both provided by the Laboratory for Parasite Genome Analysis, Cambridge University). The sequence of the entire ORF and its 5′ and 3′ flanking sequences were determined from this P1 clone by standard procedures using sequencing primers based on the partial sequence. The 5′ splice site and 3′ polyadenylation site were mapped by 5′ and 3′ nested reverse transcription-PCR (RT-PCR), respectively, as previously described (20, 25).

**Plasmid construction and transfection.** The flanking regions of *TbMP44* were PCR amplified using genomic DNA of *T. brucei* bloodstream forms (strain 427) as a template to create the construct to target replacement (knockout) of the first allele of *TbMP44.* Oligonucleotides 3322 (5′-ATAAGAATGCGGCCGCTAAACTATAGTATGGCGTTTGTAG-3′ [*Not*I site underlined]) and 3403 (5′-ACGCGTGCGGAACCGATAAAACAAG-3′ [*Mlu*I site underlined]) were used to obtain a DNA fragment containing 457 nucleotides (nt) of the 5′ flanking region of *TbMP44.* Oligonucleotides 3326 (5′-GCTCTAGAGCCGTTGAAGCGTGATTCACTC-3′ [*Xba*I site underlined]) and 3327 (5′-AAGGCCTTTGCTTCTCCTTTAACTCTATTG-3′ [*Stu*I site underlined]) were used to amplify a 3′ flanking region of *TbMP44* that is 438 nt long. Plasmid pTbMP44KO1 for this first allele knockout was derived from pLew13 (39), which contains genes for T7 RNA polymerase and the selectable marker *NEO.* The *Not*I-*Mlu*I fragment (which contains the tubulin sequence) and the *Xba*I-*Stu*I fragment of pLew13 were replaced by 5′ and 3′ flanking regions of *TbMP44,* respectively. The pTbMP44KO1 was linearized by *Not*I prior to transfection. Plasmid pTbMP44KO2 was created to target replacement of the second allele of *TbMP44* and is a derivative of pTbMP44KO1. pTbMP44KO1 was first cut by *Xba*I, and the 5′ overhang was filled using Klenow fragment (GIBCO-PRL) to form a blunt end. The linearized pTbMP44KO1 was digested with *Xho*I, and the fragment which contains 5′ and 3′ flanking regions of *TbMP44* was gel purified and ligated to the *Xho*I-*Stu*I fragment of pLew90 (39), which contains a mutant T7 promoter with 10% activity, *Tet*R, and the selectable marker *HYG.* Plasmid pTbMP44r was created to introduce a copy of *TbMP44* that is under the control of a regulatable promoter. To do this, oligonucleotides 3578 (5′-*AAGCTT*ATGAGACGGGCTGTGGTACTC-3′ [*Hin*dIII site underlined]) and 3579 (5′-*GGATCC*TTACCGCCCTCCCAGTGCCAG-3′ [*Bam*HI site underlined]) were used to amplify the entire coding region of *TbMP44* from genomic DNA. The resultant product was used to replace the *Hin*dIII-*Bam*HI fragment of the luciferase gene of pLew79 (39).

Bloodstream form *T. brucei* (strain 427) was transfected essentially as previously described (33). Briefly, $5 \times 10^7$ cells were washed in Cytomix buffer (37) and resuspended in 1 ml of Cytomix. A 10-μg portion of *Not*I-linearized plasmid DNA was mixed with 0.5 ml of cell suspension in a 0.4-cm-gap-width cuvette and electroporated. Electroporated cells were transferred to HMI-9 medium with final antibiotic concentrations of 2.5 μg of G418 per ml, 5 μg of hygromycin per ml, or 2.5 μg of phleomycin per ml. Resistant clones were obtained after 7 to 10 days. Cells that were devoid of the endogenous *TbMP44* alleles and contained an ectopic allele with its expression under the control of a *Tet*-regulatable promoter were obtained by transfection with pTbMP44KO1, pTbMP44, and pTbMP44KO2 in that order. Correct integration was confirmed by Southern analysis. The culture medium was adjusted to 1 μg of tetracycline per ml for expression of the ectopic *TbMP44* allele. Expression of *TbMP44* was inactivated by harvesting and washing a 100-ml culture of clone 2C1 log phase cells as described above and resuspending the cells in HMI-9 medium minus tetracycline at a final density of $0.5 \times 10^6$/ml. The cell number was determined at different time points, and the cumulative cell number was determined by adjusting for volume change at the time of cell transfer.

**Glycerol gradient fractionation.** A total of $6 \times 10^8$ log-phase bloodstream form *T. brucei* were lysed in 500 μl of IP buffer (10 mM Tris [pH 7.6], 10 mM $MgCl_2$, 300 mM KCI, 1% Triton X-100) containing protease inhibitors (10 μg of leupeptin per ml, 5 μg of pepstatin per ml, 1 mM Pefabloc). The lysates were cleared by a 15-min centrifugation at 13,000 rpm in an Eppendorf 5415D centrifuge at 4°C and then loaded onto 10 to 30% (vol/vol) glycerol gradients. The gradients were centrifuged at 38,000 rpm at 4°C in a Beckman SW40 rotor for 5 h as indicated. Then 500-μl fractions were collected sequentially from the top, flash-frozen in liquid nitrogen, and stored at −80°C for further analysis.

**Immunoprecipitation of editosomes.** Editosomes were immunoprecipitated as previously described (27). (MAbs) specific for editosome proteins were conjugated to anti-mouse immunoglobulin G-coated immunomagnetic beads (M-450 Dynabeads) as specified by the manufacturer (DYNAL). The beads were resuspended in 450 μl of IP buffer to which 50 μl of cleared whole-cell lysate had been added and incubated at 4°C with bidirectional mixing for 1 h. The beads were washed three times with IP buffer and once with HHE (25 mM HEPES [pH 7.9], 10 mM $MgCl_2$, 50 mM KCI, 0.5 mM EDTA) and suspended in 50 μl of HHE.

**Western analysis and adenylation.** Immunoprecipitated editosomes (10 μl) or glycerol gradient fraction (80 μl) was resolved by SDS-PAGE (10% polyacrylamide) and transferred onto a nitrocellulose filter. The filter was blocked in 5% nonfat milk powder in PBST (10 mM phosphate buffer [pH 7.2], 150 mM NaCl, 0.1% Tween 20) overnight. The filter was washed with PBST twice and incubated with a cocktail of MAbs against TbMP81, TbMP63, TbREL1, and TbMP42 (diluted 1:50) or anti-hsp70 (diluted 1:1,000) in 5% nonfat milk powder–PBST for 1 h at room temperature. The filter was then washed three times with PBST and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Bio-Rad) at 1:2,000 in 1% nonfat milk powder–PBST. After four washes with PBST, the filter was developed using an ECL kit (Amersham) as specified by the manufacturer. Adenylation of RNA editing ligases was assayed as described previously (31). The reaction products were separated by SDS-PAGE (10% polyacrylamide and the radiolabeled proteins were visualized using a PhosphorImager (Molecular Dynamics).

**Assays of RNA editing.** The presence of edited and preedited RNA was assayed by RT-PCR as previously described (33). The upstream and downstream primers for RT-PCR analysis were 3704 (5′-AAAAATAAGTATTTTGATATTATTAAAG-3′) plus 3580 (5′-TATTATTAACTTATTTGATC-3′) for ATPase subunit 6 mRNA, 3705 (5′-ATGACTACATGATAAGTA-3′) plus 3601 (5′-CGGAAGACATTGTTCTACAC-3′) for ND7, 3619 (5′-CTAATACACTTTTGATAACAAAC-3′) plus 3620 (5′-AAAAACATATCTTATATCTAAA-3′) for RPS12, and 3706 (5′-TGTGTGACTACCAGAGAT-3′) plus 3707 (5′-ATCCTATACCCGTGTA-3′) for NADH dehydrogenase subunit 4 mRNA (ND4). The PCR products were resolved on 10% polyacrylamide gels. Poisoned primer extension analysis was performed as previously described (8), except that 0.25 mM ddGTP was used instead of 0.5 mM dGTP. A 20-μg portion of total-cell RNA from each time point was mixed with 5′-labeled oligonucleotide 3566 for ND7 (5′-CACATAACTTTTCTGTACCACGATGC-3′ or 3562 for COI (5′-GTAATGAGTACGTTGTAAAACTG-3′). The reaction was stopped by the addition of phenol-chloroform-isoamyl alcohol (25:24:1 [pH 8.0]), and the ethanol-precipitated products were resolved on 9% polyacrylamide gels containing 7 M urea and visualized with a PhosphorImager.

Precleaved insertion editing was assayed using a 5′ CL18, 3′ CL13pp, and gPCA6-2A RNA tripartite substrate as previously described (14). The tripartite precleaved deletion editing substrate was U5-5′CL, U5-3′CL, and gA6[14]PC-del (15). Endoribonuclease activity was assayed using as a substrate the A6U5 RNA along with a version of its cognate gRNA that contains 3 C's in the guiding region (5). The edited products were separated on 9% polyacrylamide gels containing 7 M urea, and these were visualized and quantified using a PhosphorImager.

## RESULTS

**Identification of *TbMP44*.** Mass spectrometric analyses of editosomes that were purified by biochemical methods or immunoprecipitated by a MAb specific for TbMP63 identified a total of 11 peptides that match those predicted from an 1,146-nt ORF encoding a 44-kDa 382-amino-acid protein. This gene was designated *TbMP44* according to the naming convention (26, 27). The N-terminal portion of TbMP44 has a sequence consistent with mitochondrial targeting, and omission of this sequence predicts a protein size that matches that of the corresponding protein in SDS-PAGE gels. BLAST searches of the National Center for Biotechnology Information database did not identify any orthologous proteins, but Profilescan detected similarity to an RNase III motif. This region of similarity contains the catalytic center, in which five of nine consensus residues are conserved as well as the E and G that function in the *Escherichia coli* enzyme in substrate binding and Mg enhancement of catalysis, respectively (reference 22 and data not shown). Thus, TbMP44 is a novel protein with some similarity to *E. coli* RNase III, which suggests that it may interact with RNA and perhaps have nucleolytic activity. However, none of the catalytic activities associated with editing, including endoribonuclease, 3′ TUTase, 3′ exoUase, and RNA ligase, were detected with in vitro-transcribed-translated TbMP44 that was precipitated via an N-terminal $His_6$ tag (data not shown). While protein was produced in vitro and this approach allowed the detection of TbREL1 and TbREL2 ligase activities (33), there may not have been sufficient active protein produced and/or precipitated to detect other activities, or, alternatively, TbMP44 may not play a catalytic role or may play one that is different from the ones assayed.

**TbMP44 is essential for growth of bloodstream forms.** Northern analysis of procyclic form RNA (Fig. 1A, right panel) revealed a ~1.3-kb TbMP44 transcript, and the 5′ spliced leader attachment and 3′ polyadenylation sites were mapped by nested RT-PCR to nt −24 and 1392, respectively. Southern analysis shows that *TbMP44* is a single-copy gene (right panel). One of the *TbMP44* alleles was disrupted in bloodstream forms by targeted gene replacement (39) with a T7 RNA polymerase gene and neomycin (*NEO*) resistance selectable marker, as diagrammed in Fig. 1A and described in Materials and Methods. Several attempts to replace the second allele were unsuccessful, suggesting that TbMP44 may be essential for survival. Hence, a tetracycline (tet) regulatable ectopic copy of *TbMP44* was inserted into the ribosomal DNA spacer region and then the second *TbMP44* allele was replaced with the tet repressor (*TetR*) gene and a hygromycin (*HYG*) resistance selectable marker (Fig. 1A, left panel). Selection and cloning were done in the presence of tet to enable expression of the ectopic allele. Three clones were obtained with the desired integrations as determined by Southern analysis (right panel), including clone 2C1, which was chosen for further analysis. Wild-type levels of *TbMP44* mRNA were present in clone 2C1 in the presence of tet, but the level of this RNA was dramatically reduced within 24 h following the removal of tet from the culture medium (Fig. 1B). Following the removal of tet, clone 2C1 cells continued to grow for about 72 h at a rate comparable to that of cells in the presence of tet (or of wild-type cells). After this time, movement of the cells became sluggish and cell proliferation ceased, until more than 90% of the cells were dead after 144 h (Figure 1C). However, reintroduction of tet 72 h after its withdrawal resulted in a resumption of cell growth at a rate similar to that of the culture from which tet had not been withdrawn. Thus, TbMP44 expression is essential for the growth and survival of bloodstream form *T. brucei.*

**Inactivation of TbMP44 disrupts RNA editing.** The effect of inactivation of *TbMP44* gene expression on RNA editing in vivo was assessed by RT-PCR and primer extension analysis of total-cell RNA from the clone 2C1 bloodstream forms that was isolated at 24, 48, 72, and 96 h after removal of tet (Fig. 2).

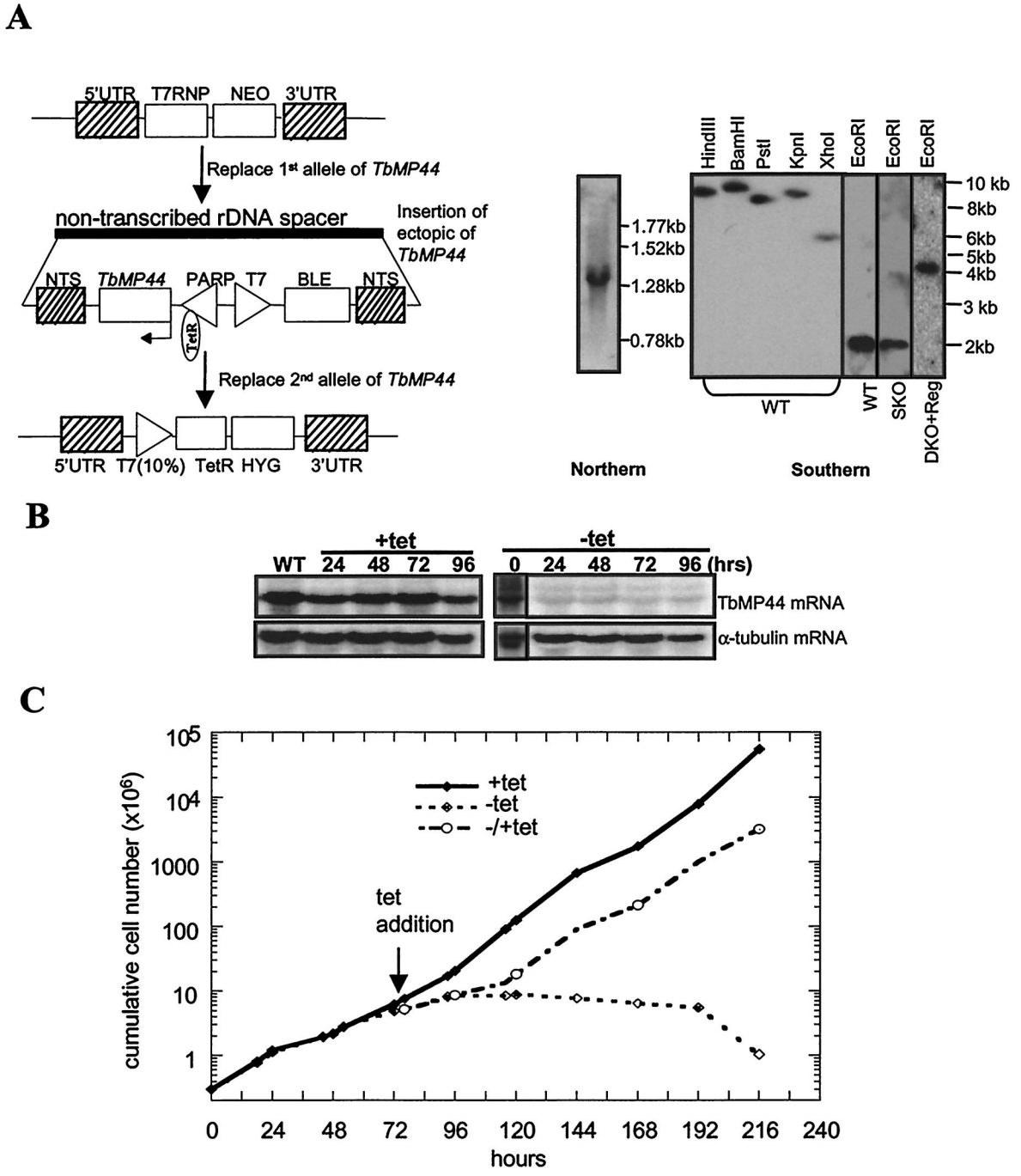



FIG. 1. Inactivation of *TbMP44* gene expression. (A) The left panel shows the strategy for replacement of endogenous *TbMP44* alleles and introduction of a regulatable ectopic allele. Sequences for targeting recombinational replacement (UTR and NTS) are shaded. Constructs were prepared with neomycin (*NEO*), phleomycin (*BLE*), or hygromycin (*HYG*) selectable markers, linearized by *Not*I, and sequentially transfected into bloodstream form *T. brucei* as described in Materials and Methods. Clone 2C1 was confirmed as having the desired genotype. The right panel shows Northern analysis of total RNA and Southern analyses of DNA from wild-type procyclic *T. brucei* 427 (WT) cells and from bloodstream forms of wild-type, transgenic single-knockout (SKO), and transgenic double-knockout with regulatable T*bMP44* allele (DKO + Reg) strains using probes corresponding to the coding region of *TbMP44,* as described in Materials and Methods. RNA and DNA size marker (GIBCO-BRL) positions are indicated. (B) Northern analysis using the same probe as in Fig. 1A, showing the presence or loss of *TbMP44* mRNA in clone 2C1 at various times in the presence of 1 μg of tet per ml (+tet) or after its removal (−tet). Reprobing for α-tubulin mRNA was used as a loading control. (C) Growth of clone 2C1 cells in the presence (solid diamonds) or absence (open diamonds) of tet, or after reintroduction of tet 72 h following its removal (open circles). Cell density was maintained between $0.6 \times 10^6$ and $\sim 1.5 \times 10^6$/ml, and the cumulative cell number was normalized according to the dilution factor.

There was a progressive reduction in the abundance of RT-PCR products from fully and partially edited ND7 RNA, A6 RNA, and RPS12 RNA, while PCR products from ND4 mRNA, which does not get edited, remained similar in abundance (Fig. 2A). Pre-edited A6 and RPS12 RNA were more abundant after the removal of tet, probably reflecting their accumulation due to the lack of editing, but this does not appear to be the case for ND7 RNA. Poisoned primer extension of ND7 RNA, which allows for quantitation, revealed ~30 to 40% reduction in edited ND7 RNA after 24 to 48 h, 70% reduction after 72 h, and nearly complete absence after 96 h, all relative to 0 time, while the amount of COI RNA, which does not get edited, remained the same (Fig. 2B). Overall, these results indicate that TbMP44 is essential for RNA editing in bloodstream form *T. brucei.*

In vitro editing activities that normally sediment at ~20S were essentially absent from clone 2C1 after 72 h in the absence of tet. The endonuclease activity that cleaves a double-stranded editing substrate was essentially absent after 72 h without tet (Fig. 3A). Similarly, precleaved insertion editing, which entails TUTase and ligase reaction pairs, and precleaved deletion activity, which entails exoUase and ligase reaction pairs (14, 15), were essentially absent from clone 2C1 cells after 72 h of growth in the absence of tet (Figs. 3B and C). Some ligated product and a trace of edited product were detected by the precleaved insertion editing assay, but neither of these products was detected by the precleaved deletion editing assay. Substantial exonuclease activity was detected in the precleaved deletion assay and resulted primarily in −3U product. This is probably due to nucleases other than the editing exoUase in the whole-cell lysates since, as shown in Fig. 3D, fractions 9 to 13 from cells grown with tet primarily removed a single U from the substrate ending in UUAU, reflecting the U specificity of the editing exoUase. However, fractions from cells grown without tet did not stop at the A in the UUAU substrate but primarily removed 3 nucleotides. The absence of editosomes may have left the substrates available for nucleases other than the editing exoUase. The loss of the catalytic activities associated with editing further confirms the role of TbMP44 in editing.

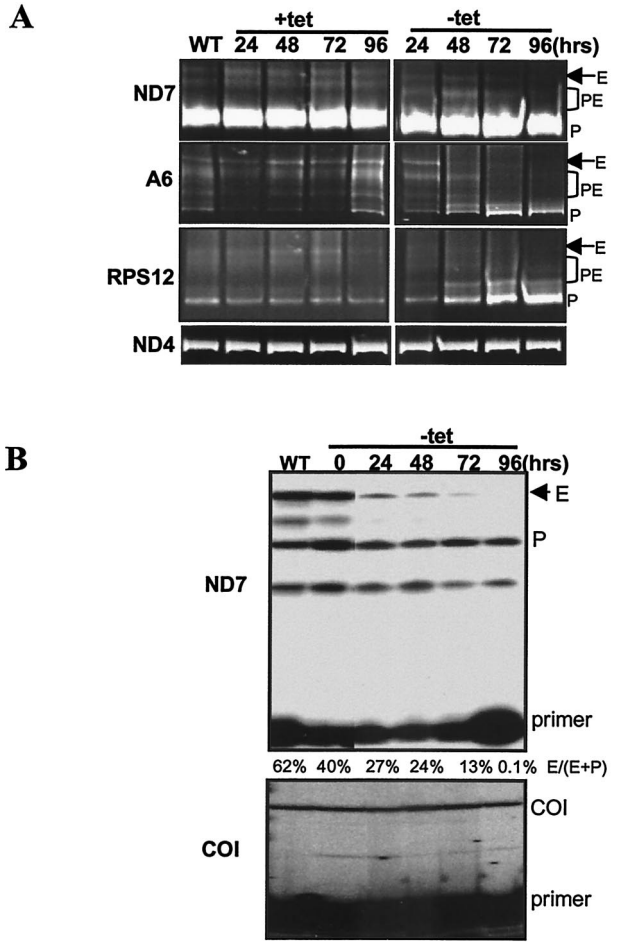



FIG. 2. Effect of inactivation of *TbMP44* expression on in vivo RNA editing. (A) PAGE analysis of RT-PCR products of ND7, ATPase 6 (A6), and RPS12 mRNAs at various times in the presence of or after removal of tet. PCR products corresponding to edited (E), partially edited (PE), and pre-edited (P) RNAs are indicated. Never-edited ND4 mRNA was used as a control. (B) Poisoned primer extension analysis of ND7. The percentage of edited ND7 RNA, calculated as E/(E + P), is shown below each lane, and never-edited COI RNA was used as a control.

**Inactivation of TbMP44 expression disrupts editosomes.** Western and adenylation analyses of glycerol gradient fractions from whole-cell lysates revealed that editosomal proteins were essentially absent from clone 2C1 cells after 72 h of growth in the absence of tet (Fig. 4). TbMP81, TbMP63, TbREL1, TbREL2, and TbMP42 proteins cosediment at ~20S from wild-type and clone 2C1 cells grown in the presence of tet (Fig. 4). However, these five editosome proteins were dramatically reduced in abundance following the inactivation of the *TbMP44* gene. Signal corresponding to low-molecular-weight proteins was detected at the top of the gradient, suggesting the presence of degraded proteins. Reprobing of the filters with anti-hsp70 antibody revealed that similar amounts of sample were loaded on these glycerol gradients. Thus, the loss of editing is paralleled by the loss of editosomal proteins and hence of editosomes.

Northern analysis of whole-cell RNA revealed that the mRNA for another editosome protein, TbMP63, was retained for at least 72 h following the removal of tet and at levels that were essentially the same as before the removal of tet (Fig. 5). However, the level of TbMP63 protein was substantially diminished within 24 h after the removal of tet and the protein was essentially gone after 72 h. In addition, the level of another editosome protein, TbREL1, was similarly reduced after 24 h and was very low by 72 h. The *TbMP44* mRNA level dropped quite rapidly after removal of tet. It was significantly diminished by 2 h, and the mRNA was essentially absent by 4 h (data not shown). TbMP44 protein could not be examined directly due to the lack of a specific antibody. In contrast, while the level of TbMP63 mRNA remained essentially unchanged for 72 h following removal of tet, the level of TbMP63 protein decreased by about 50% within 24 h and then by about 90% within 72 h (data not shown). TbREL1 protein levels decreased with a similar pattern (Fig. 5).

Whole-cell lysates of clone 2C1 that were prepared at various times after withdrawal of tet were fractionated by glycerol gradient sedimentation to assess the consequences of inactivation of *TbMP44* expression on editosome integrity. Western analyses using a mixture of four MAbs, which are specific for TbMP81,

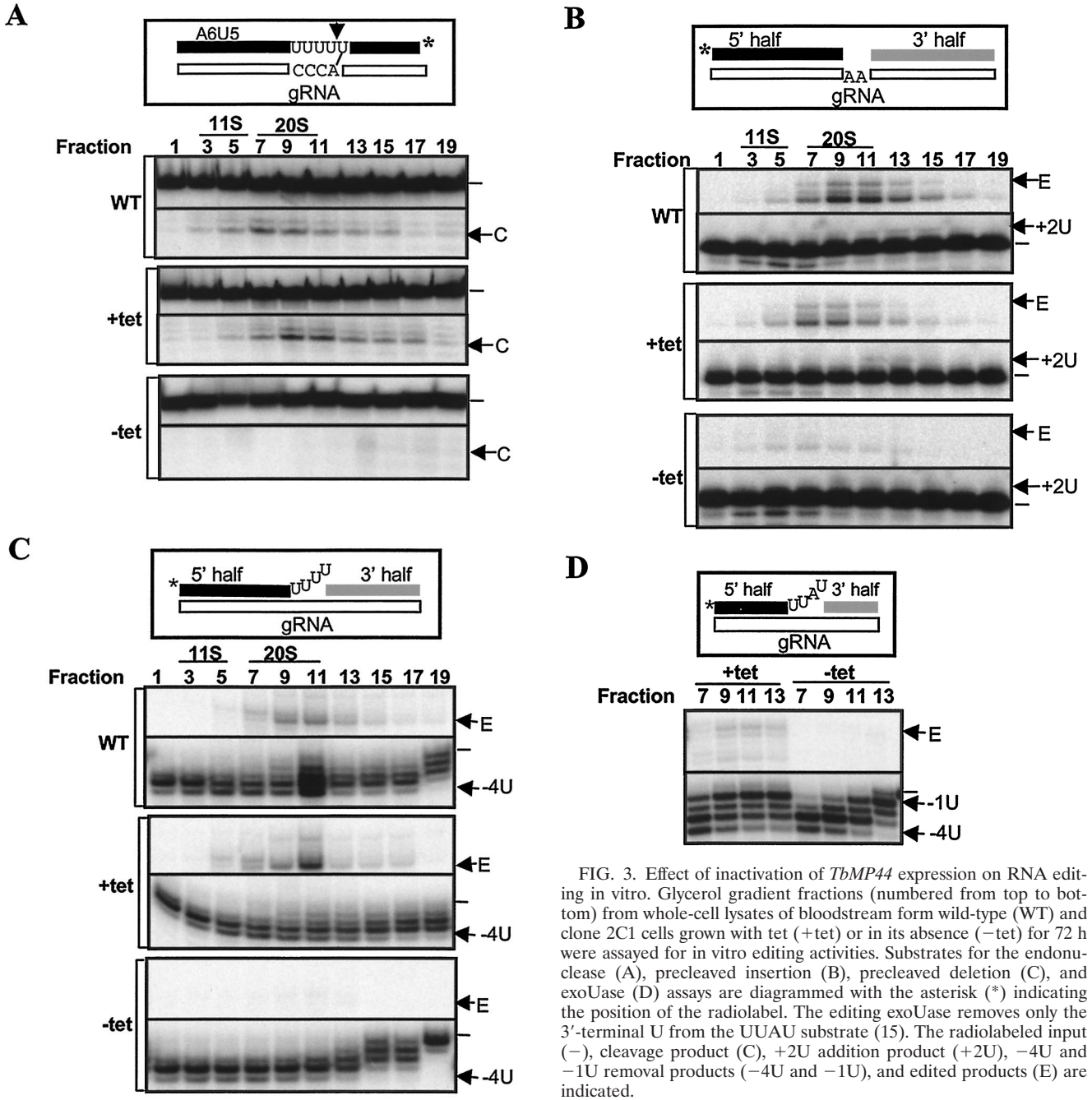
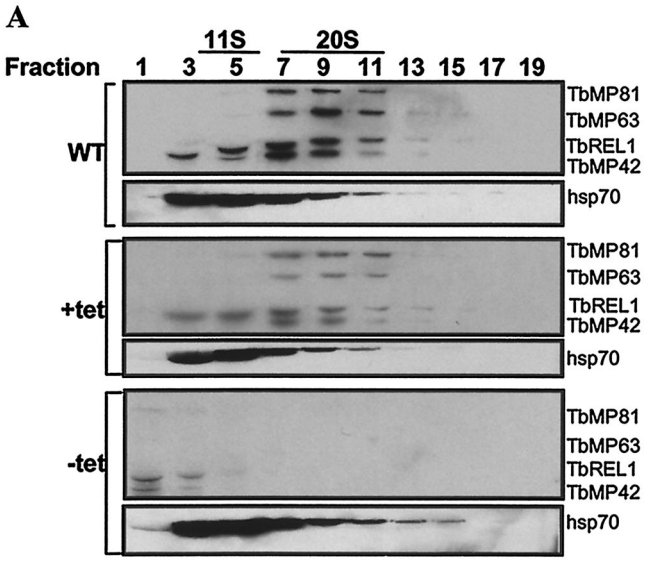



FIG. 3. Effect of inactivation of *TbMP44* expression on RNA editing in vitro. Glycerol gradient fractions (numbered from top to bottom) from whole-cell lysates of bloodstream form wild-type (WT) and clone 2C1 cells grown with tet (+tet) or in its absence (−tet) for 72 h were assayed for in vitro editing activities. Substrates for the endonuclease (A), precleaved insertion (B), precleaved deletion (C), and exoUase (D) assays are diagrammed with the asterisk (*) indicating the position of the radiolabel. The editing exoUase removes only the 3′-terminal U from the UUAU substrate (15). The radiolabeled input (−), cleavage product (C), +2U addition product (+2U), −4U and −1U removal products (−4U and −1U), and edited products (E) are indicated.

TbMP63, TbREL1, and TbMP42, were performed on the gradient fractions, as were adenylation assays, which detect both TbREL1 and TbREL2 (Fig. 6). The editosomes appear intact after 4 h following the withdrawal of tet, since the Western and adenylation results were indistinguishable from those with zero-time and wild-type cells (compare to Fig. 4). However, most of the editosome proteins progressively shifted to the ~10S fraction over 24 h. At 36 h, a substantial portion of these proteins were in the ~10S fractions but the proportion of proteins in the ~20S fractions had increased. After this time, there was a progressive diminution of the protein levels until they were barely detected after 72 h. During this time, hsp70 was readily detectable and its position in the gradient remained similar. Reexpression of *TbMP44* by the addition of tet 72 h after its withdrawal resulted in the reappearance of the editosome proteins by 48 h. Examination of cells 72 h after reexpression of *TbMP44* revealed proteins distributed primarily between 10S and 20S. These results indicate that TbMP44 is important for editosome integrity.

## DISCUSSION

This study shows that TbMP44 is a component of the *T. brucei* editosome and is required for editing and for editosome structural integrity. Repression of *TbMP44* expression inhib-



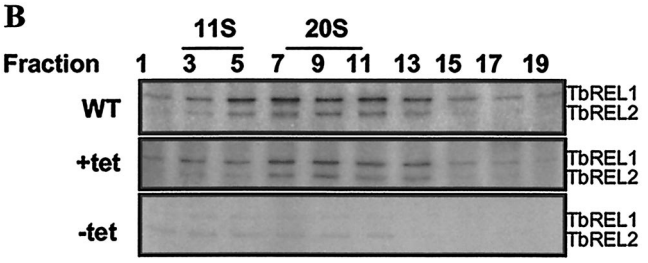




FIG. 4. Western analysis (A) and adenylation assays (B) of glycerol gradient fractions (fraction 1 at the top) from wild-type (WT) cells and clone 2C1 cells grown with tet (+tet) or in its absence (−tet) for 72 h. The Western analyses used a mixture of MAbs specific for the editosome proteins indicated, and the filters were stripped and reprobed with anti-hsp 70 MAb as a loading control. Thyroglobulin (19S) and catalase (11S) were used as sedimentation markers in parallel gradients. See Materials and Methods for experimental details for the Western and adenylation assays.

ited the growth of bloodstream forms of *T. brucei* and resulted in loss of editing in vivo, loss of editing-associated catalytic activities in vitro, and perturbation of the editosome structure. *TbMP44* mRNA essentially disappeared between 2 and 4 h after repression of *TbMP44* gene expression (data not shown). The mRNA for another editosome protein, TbMP63, remained at apparently normal levels for at least 72 h after *TbMP44* gene repression. However, this protein, other editosome proteins, and editosomes diminished in abundance until they were essentially absent 72 h after *TbMP44* gene repression. This was accompanied by a reduction in the sedimentation coefficient of editosomes. Reexpression of *TbMP44* by reintroduction of tet 72 h after repression by tet removal resulted in a normal growth rate of the culture after a ~48-h lag and reappearance of editosomes. These observations indicate that TbMP44 is essential for functional editosomes and their structural integrity.

The identification of TbMP44 increases the number of proteins that have been demonstrated to be part of the editosome.

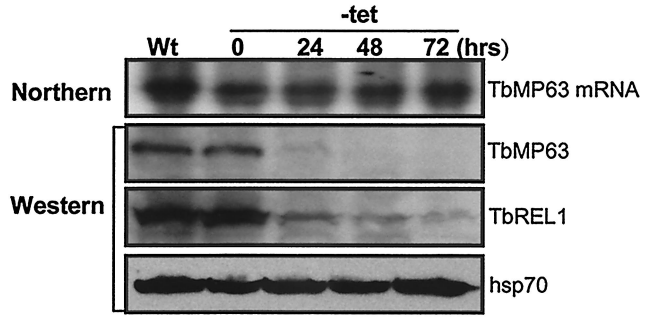



FIG. 5. Loss of editosomes upon inactivation of *TbMP44* expression. Northern and Western analyses were performed using total-cell RNA or protein. See Materials and Methods for experimental details. Samples were taken from wild-type (WT) cells and from cells grown in the absence of tet (−tet) for the times indicated. Total-cell RNA or protein was taken at 24-h intervals.

To date, over 20 proteins have been identified as candidate components of the editosome (25a). These include the TbREL1 (TbMP52) and TbREL2 (TbMP48) RNA-editing ligases (27), the mHel61p helicase (23), and TbMP63 and TbMP81, which are zinc finger proteins (27) with no known catalytic function but which are essential for the retention of TbREL1 and TbREL2, respectively (7, 13). The 108-kDa 3′ TUTase is essential for editing but has not yet been demonstrated to be in the editosome; however, TbMP57, which also has 3′ TUTase activity, is present in the editosome (Ernst et al., submitted). Hence, these proteins may catalyze 3′ U addition to the gRNA and to pre-mRNA, respectively. The editing endonuclease(s) and the 3′ exoUase have not been identified. Other proteins, such as gBP21 (1, 16) and gBP25 (4), RBP16 (11), TbRGG1 (38), and REAP-1 (19), all of which appear to be RNA binding proteins, may play roles in editing, perhaps as accessory factors.

The specific function of TbMP44 is uncertain, but it is clearly essential for editosome integrity. It contains an RNase III-like motif, which is conserved in its *T. cruzi* and *Leishmania major* orthologs (Salavati et al., unpublished), suggesting that it may be an endonuclease. We could not detect endonuclease or other catalytic activities with in vitro-transcribed-translated protein. Such activities may have been below the sensitivity of the assays due to inadequate protein levels, or its function may require or be enhanced by the presence of other proteins which we have observed for other editing catalysts. Alternatively, TbMP44 may retain some characteristics of an endonuclease, such as RNA binding. Loss of TbMP44 does not preferentially affect RNA deletion or insertion editing; rather, all activities are lost, as is editosome structural integrity.

The requirement of TbMP44 for the structural integrity of the editosome implies a role in editosome stability and/or assembly. About 50% of TbREL1 and TbMP63 editosome proteins are lost within 24 h of inactivation of TbMP44 expression. TbMP44 mRNA is lost within 2 to 4 h, and TbMP44 protein levels then must drop to levels that affect function, perhaps within a few hours. Thus, the generation time of ~13.5 h for 2C1 cells suggests that they underwent about one generation between the time when the TbMP44 protein level became critical and the time when about half of the editosome proteins

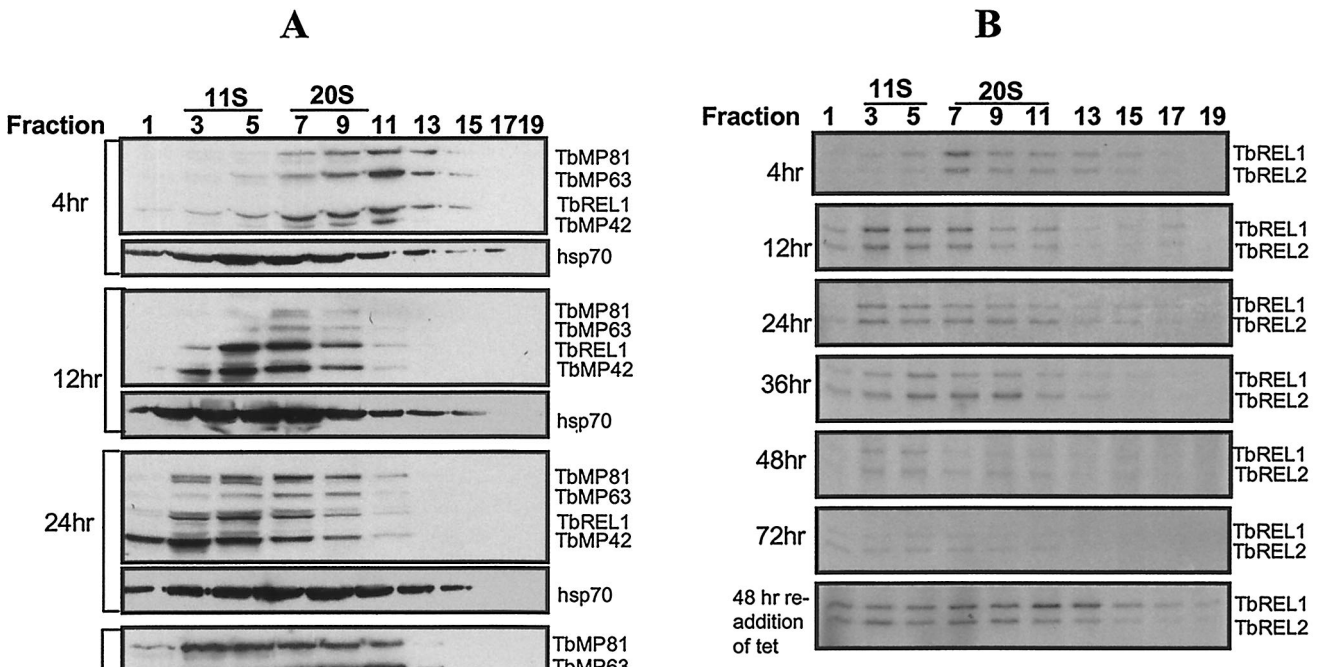



FIG. 6. Editosome changes resulting from inactivation of *TbMP44* expression. Glycerol gradient fractions of whole-cell lysates from samples were taken after the removal of tet at the times indicated and 48 h after the addition of tet to cells at 72 h after removal of tet. The gradient fractions were assayed by Western analysis using a mixture of four MAbs specific for the TbMP81, TbMP63, TbREL 1, and TbMP42 editosome proteins (A) and by adenylation that detects the TbREL1 and TbREL2 editing ligases (B). The filters were stripped and reprobed with anti-hsp70 MAb as a control. See Materials and Methods for details.

were lost. This suggests that there was no net gain or loss of these editosome proteins during this time interval. It implies that either no newly synthesized proteins were incorporated into editosomes or incorporation of newly synthesized proteins was in balance with their rate of turnover. We cannot distinguish between these alternatives at this time, and hence we cannot tell whether TbMP44 plays a role in de novo assembly of editosomes and/or their stability. However, the appearance of minor bands and changes in relative intensity among the bands in the Western analyses (Fig. 6) imply that some protein degradation is occurring within the 24-h period.

The shift of most editosomes from ~20S to ~10S by 24 h, indeed by 12 h when the levels of TbREL1 and TbMP63 were reduced by only ~25%, suggests the involvement of a more dynamic process than de novo assembly and/or editosome protein turnover. Such a process might entail a dissociation of editosome subunits and/or accessory factors. This shift is intriguing in view of the accumulating evidence that insertion editing and deletion editing are functionally and physically separate. This separation was first implied by the presence of two editing ligases (21, 27, 29, 31) that have somewhat distinct catalytic characteristics (6, 13, 30). It is further supported by the preferential loss of TbREL2 upon TbMP81 gene inactivation (7) and of TbREL1 upon TbMP63 gene inactivation (13). Indeed, recent studies indicate the existence of editosome subunits for insertion and deletion editing (A. Schnaufer et al., submitted for publication). These studies strongly support the separation of these two types of editing. One possibility that we suggest is that the insertion and deletion editosome subunits separate during the editing process, perhaps after each gRNA is used, and that TbMP44 is essential to the reassembly with another gRNA. These possibilities are currently under assessment.

The reproducible relative increase in the level of ~20S editosomes 24 h after *TbMP44* gene inactivation, which is more evident at the 36-h time point, implies the occurrence of a second process. One possibility is that another protein may substitute for the assembly, stability, or subunit interaction function of TbMP44. A candidate for this substitution is TbMP46, which has significant sequence identity and similarity

to TbMP44 (25a). Such a substitution may not result in functional editosomes and hence may ultimately lead to editosome loss and cell death. An alternative possibility is that the shift to a larger S value might reflect an association with protein-processing machinery. Overall, the repression of TbMP44 expression results in the eventual diminution and loss of editosomes and editosome proteins. Thus, despite the specific role of TbMP44, it is essential for the production and/or retention of editosomes.

The rescue of cells from death and the reacquisition of editosomes upon reactivation of *TbMP44* gene expression after 72 h indicate that the temporary loss of editosomes was not lethal and that the loss of editosomes is due to inactivation of *TbMP44* gene expression. The difference in the sedimentation profile of the editosome proteins in the rescued cells compared to the zero-time and wild-type cells may reflect the presence of dead or dying cells in the 72-h sample and/or the presence of partially assembled editosomes that follow the lag in returning to the normal growth rate following reactivation of *TbMP44* gene expression.

The inhibition of growth of bloodstream forms upon inactivation of *TbMP44* expression supports the suggestion that editing is normally required in bloodstream forms (33). Inactivation of other editosome protein genes, including the TbREL1 editing RNA ligase (33) and the TbMP81 (7) zinc finger protein, also is lethal in bloodstream forms, as is inactivation of mt topoisomerase II, which creates mutants lacking mt DNA (32). This reduces the possibility that editosome proteins are multifunctional and that lethality is due to functions not associated with editing. The finding that editing is normally essential in bloodstream forms suggests that the significance of mt gene expression in this life cycle stage, e.g., for generation of ATP (10) and other potentially vital functions, is incompletely understood. These forms lack cytochromes and were thought to rely solely on glycolysis for ATP production. There is no evidence that glycolytic enzyme production requires RNA editing. However, NADH dehydrogenase complex and/or ATPase complex, both of which reside in the inner mt membrane and contain subunits translated from edited mRNA, may have functions essential for the bloodstream stage. Mutants that do not edit normally, if at all, since they lack all or most kDNA, appear to have compensated for this loss (6a, 32).

Overall, TbMP44 was shown to be a component of the editosome that is critical for editosome function and editosome assembly and/or structural integrity. No catalytic function was identified, but it may play a role in dynamic processes that occur during editing. It is normally vital for bloodstream form *T. brucei* and has homologs in other pathogenic trypanosomatids and thus may be a potential drug target for chemotherapy for African sleeping sickness and related diseases.

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