Bloodstream form *Trypanosoma brucei* do not require mRPN1 for gRNA processing

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ABSTRACT

Mitochondrial RNA processing in the kinetoplastid parasite *Trypanosoma brucei* involves numerous specialized catalytic activities that are incompletely understood. The mitochondrial genome consists of maxicircles that primarily encode rRNAs and mRNAs, and minicircles that encode a diverse array of guide RNAs (gRNAs). RNA editing uses these gRNAs as templates to recode mRNAs by insertion and deletion of uridine (U) residues. While the multiprotein complex that catalyzes RNA editing has been extensively studied, other players involved in mitochondrial RNA processing have remained enigmatic. The proteins required for processing mitochondrial polycistronic transcripts into mature species was essentially unknown until an RNase III endonuclease, called mRPN1, was reported to be involved in gRNA processing in procyclic form parasites. In this work, we examine the role of mRPN1 in gRNA processing in bloodstream form parasites, and show that complete elimination of mRPN1 by gene knockout does not alter gRNA maturation. These results indicate that another enzyme must be involved in gRNA processing.

Keywords: guide RNA; kinetoplastid; RNA editing; RNA processing; RNase III

INTRODUCTION

Decades of research has led to numerous insights into mitochondrial RNA processing in *Trypanosoma brucei* and related kinetoplastid pathogens, with a significant focus on RNA editing, the process that recodes mRNAs by the insertion and deletion of uridine residues (Us) (Stuart et al. 2005; Hajduk and Ochsenreiter 2010; Aphasizhev and Aphasizheva 2011, 2014; Göringer 2012). Editing can be extensive, and is ultimately responsible for the majority of the coding sequence in some transcripts (Feagin et al. 1988). RNA editing is catalyzed by editosomes, multiprotein complexes that contain endonuclease, terminal uridylyltransferase, exonuclease, and ligase activities. Three distinct editosomes have been identified, each typified by a mutually exclusive and essential RNase III-type endonuclease (KREN1, KREN2, or KREN3) (Panigrahi et al. 2006; Carnes et al. 2008). Together these endonuclease-specific editosomes somehow recognize numerous similar, but distinct editing sites that are defined by template gRNAs partially annealed to preedited mRNAs. After endonucleolytic cleavage of the mRNA at the editing site, Us are either inserted or removed to make the mRNA sequence complementary to the gRNA, thus transferring the sequence information from gRNA to mRNA. For extensively edited mRNAs, multiple gRNAs are required to produce a mature transcript. Recent gRNA sequencing efforts have identified >1200 distinct gRNAs involved in procyclic (insect) stage RNA editing, highlighting the complexity of the process (Koslowsky et al. 2014). Life cycle-dependent changes in RNA editing add yet another layer of complexity, and while the mechanism behind this developmental regulation is unknown, it does not appear to result from differences in gRNA expression (Koslowsky et al. 1992; Schnaufer et al. 2002). Instead, it has been hypothesized that developmental regulation occurs via differential gRNA utilization, although this supposition (like other aspects of gRNA processing) is lacking in direct experimental evidence.

Despite extensive research on RNA editing, existing evidence paints an incomplete picture of how primary gRNA transcripts are processed into mature gRNAs. Almost all gRNAs are encoded on minicircles, which in *T. brucei* typically encode 3–5 gRNAs, with each flanked by 18-bp inverted repeats. The inverted repeat at the 5′ end is thought to be involved with transcription initiation, typically at a downstream conserved 5′-ATATA-3′ sequence (Pollard et al. 1990; Koslowsky et al. 2014). Transcription is polycistronic, and the 5′ triphosphate detected on gRNA precursors

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suggests that transcription initiation at the 5′ end of each gRNA sequence creates the mature 5′ end on most (if not all) gRNAs, while a presumed 3′ endonucleolytic cleavage event separates the 3′ end of the gRNA from rest of the polycistron (Blum and Simpson 1990; Blum et al. 1990; Grams et al. 2000; Clement et al. 2004; Madina et al. 2011). RNA sequence or structural motifs that signal for the 3′ processing event have not been reported. Similarly, the fate of the released 3′ portion of the polycistron is unknown, including whether any of the gRNA sequences contained therein are ever processed into mature gRNAs. In vitro cleavage of gRNA polycistronic precursors was initially detected in mitochondrial lysates, cosedimenting with ~20S editosome, but the source of this activity was not identified (Grams et al. 2000). Subsequently, a mitochondrial RNase III-type endonuclease called mRPN1 (mitochondrial RNA precursor-processing endonuclease 1) was characterized as cleaving gRNA polycistrons in procyclic cells (see below). Post-transcriptional addition of the poly(U) tail found on mature gRNAs is primarily attributed to KRET1 activity, although several other terminal uridylyltransferases (TUTases) exist (Blum and Simpson 1990; Blum et al. 1990; Aphasizhev et al. 2002). Uridylylation by KRET1 is required for nucleolytic processing of gRNA precursors, and it has been suggested that antisense transcripts (another target of KRET1 activity) direct nucleolytic processing of gRNA polycistrons (Aphasizheva and Aphasizhev 2010).

Identification of mRPN1 stemmed from its sequence similarity to the editing endonucleases KREN1-3 (Madina et al. 2011). Although conservation of the U1-like zinc-finger and RNase III motifs has been maintained among these proteins, the putative double strand RNA Binding Motif (dsRBM) in the editing endonucleases has recently been shown to be a PUF domain (Carnes et al. 2012). In contrast, mRPN1 appears to maintain an identifiable dsRBM. Unlike the editing endonucleases, mRPN1 forms homodimers, and does not associate with known editosome proteins (Carnes et al. 2011; Madina et al. 2011). In vitro cleavage by mRPN1 is reminiscent of that by RNase III, leaving 2-nucleotide 3′ overhangs on dsRNA substrates, requiring Mg^{2+} and a conserved catalytic aspartate for activity. Knockdown of mRPN1 by RNAi in procyclic cells generated a significant growth defect, and concomitant partial loss of total gRNAs detected by guanylyltransferase labeling, as well as loss of specific gRNAs by northern blot (Madina et al. 2011). Real-time PCR analysis also showed that mRPN1 loss was also associated with an increase in gRNA polycistronic precursors, a decrease in fully edited RNAs, and no effect on mRNA polycistronic precursors. mRPN1 was also found to associate with components of the MRB1 complex, particularly TbRGG2, even though previous examinations of the MRB1 complex did not detect mRPN1 (Hashimi et al. 2008, 2013; Panigrahi et al. 2008; Weng et al. 2008; Acestor et al. 2009; Hernandez et al. 2010; Madina et al. 2011). While the precise functions of the MRB1 complex are not yet clear, it is thought to facilitate gRNA exchange when editosomes require multiple gRNAs for editing, and to also coordinate RNA editing with other RNA processing events (Hashimi et al. 2013). Together, these data implied that mRPN1 was an endonuclease required for normal processing of polycistronic gRNA precursors.

In this work, we describe the consequences of eliminating mRPN1 expression in bloodstream form *T. brucei*. In contrast to previously reported RNAi-mediated knockdown of mRPN1 in procyclic form cells, we find little growth defect resulting from the absence of mRPN1, no defect in the total gRNA population, and no defect in the processing of specific gRNA polycistrons. These unexpected results suggest the potential for differences in gRNA processing between life cycle stages and indicate the existence of additional mechanisms for gRNA processing that have yet to be discovered.

RESULTS AND DISCUSSION

To determine the role that mRPN1 plays in gRNA processing in bloodstream form *T. brucei*, both *mRPN1* alleles were eliminated by homologous recombination to generate mRPN1 null cells. Similarly, mRPN1 conditional null cells were generated by the introduction of a tetracycline-regulated, TAP-tagged *mRPN1* allele into the *rDNA* locus in the mRPN1 null background. To demonstrate the elimination of *mRPN1*, genomic DNA was isolated from cells and analyzed by PCR (Fig. 1). Four different primer pairs (generating ORF1-4) were tested to detect all or parts of the *mRPN1* coding sequence, using combinations of forward and reverse primers in the 5′ and 3′ UTRs, as well as forward and reverse primers in the ORF. As expected, PCR products for *mRPN1* were detected in DNA from 427 wt control cells, but not in DNA from mRPN1 null cells. Similarly, PCR products for *mRPN1* that required the endogenous UTRs were not observed in mRPN1 conditional null cells, while the primers recognizing *mRPN1* ORF sequence were able to detect the tetracycline-regulated ectopic allele in the *rDNA* locus, as expected. Additional PCR analyses were performed to demonstrate that the knockout constructs had replaced *mRPN1* as expected. These PCRs used primers that flank the UTR sequences used for homologous recombination, with one primer in the *mRPN1* locus and the other in the knockout construct for each primer set. Amplicons indicating the expected integrations at the 5′ and 3′ junctions for the first and second knockout constructs were observed in PCRs using DNA from mRPN1 null and conditional null cell lines, but not in DNA from 427 wt control cells. These results show that both *mRPN1* alleles have been eliminated in mRPN1 null and mRPN1 conditional null cell lines. More importantly, the creation of an mRPN1 null cell line demonstrates that this gene is not essential in bloodstream form parasites. The dispensability of mRPN1 in bloodstream form parasites is an unexpected result considering the apparent essentiality of mRPN1 in procyclic forms and the requirement

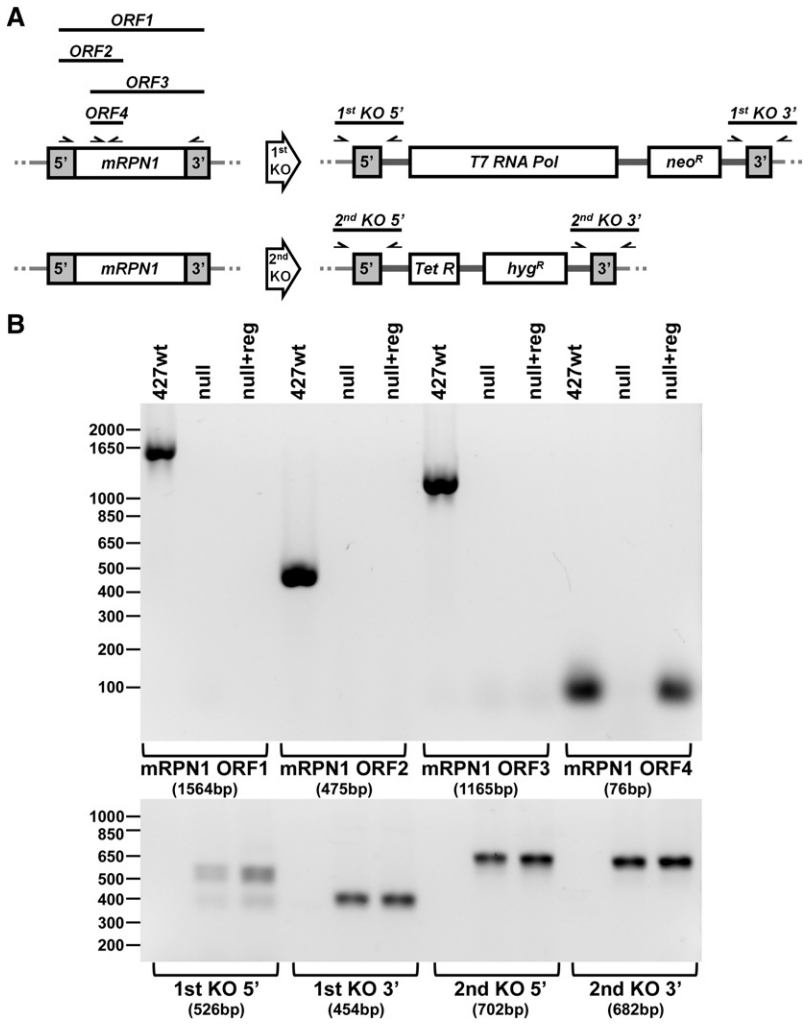

FIGURE 1. PCR analyses demonstrate elimination of the *mRPN1* coding sequence. (*A*) A schematic of the two *mRPN1* alleles, the drug-resistance cassettes used to replace them by homologous recombination, and the locations of primers (small arrows) and resulting PCR amplicons (black bars) used to analyze cell lines for *mRPN1* knockout. Gray boxes labeled 5′ and 3′ represent the UTR sequences used for homologous recombination. Four different primer pairs were used to amplify portions of the *mRPN1* open reading frame (called ORF1–4), with relative sizes depicted by black bars. Also shown are the four primer pairs used to detect integration of the drug-resistance cassettes at the 5′ and 3′ ends of the first and second knockout (KO) constructs. (*B*) Agarose gel analyses of PCR products from parental 427 wt, mRPN1 null (null), and mRPN1 conditional null (null + reg) cell lines. The *top* gel shows *mRPN1* amplicons for ORF1–4, with expected amplicon sizes indicated. The *bottom* gel shows integration amplicons for the 5′ and 3′ ends of each knockout construct, with expected amplicon sizes indicated. Migration of 100-bp ladder indicated on the *left* side of each gel in base pairs.

for RNA editing in both life cycle stages (Schnaufer et al. 2001; Madina et al. 2011).

To assess the consequences of mRPN1 loss on parasite growth, we compared mRPN1 null cells to parental 427 wt cells, and mRPN1 conditional null cells with mRPN1 either expressed or repressed (Fig. 2). The cumulative cell numbers of 427 wt appeared to be marginally greater than mRPN1 null; however, the variation observed is similar in extent to what can sometimes be observed among a series of cell lines following transfection. In other words, it is not clear whether this slight growth difference is a functional consequence of the loss of mRPN1 or merely a spurious consequence of cloning a cell line following transfection. Nevertheless, a similar slight growth defect is observed in mRPN1 conditional null cells when mRPN1 is repressed. Repression of mRPN1 was assessed by Western analysis of cell lysates using recombinant peroxidase anti-peroxidase, which binds to the Protein A moiety of the TAP tag fused to the ectopic mRPN1 allele. The Western result clearly demonstrates that the regulated mRPN1-TAP protein is undetectable in repressed cells over 3 d. Together, these data suggest that the loss of mRPN1 produces a slight defect in growth in bloodstream form cells. In contrast, RNAi-mediated repression of mRPN1 in procyclic cells produces a strong and persistent growth defect (Madina et al. 2011). Expression knockdown using the conditional null approach is typically more complete than the knockdown achieved by RNAi, further highlighting the differences observed in procyclic and bloodstream form results (Merritt and Stuart 2013).

Because loss of mRPN1 has been shown to decrease the amount of gRNAs in procyclic cells, we examined the total gRNA population in bloodstream form cells lacking mRPN1 (Fig. 3). To visualize gRNAs, total RNA was extracted and subjected to labeling with guanylyltransferase and [γ-^{32}P]ATP. Guanylyltransferase labeling takes advantage of the 5′ triphosphate of gRNAs, and produces a typical series of bands reflecting both sequence variability and U-tail heterogeneity (Blum and Simpson 1990; Aphasizheva and Aphasizhev 2010; Madina et al. 2011). Guanylyltransferase also labels other larger RNAs in extracts from *T. brucei*, which provide a convenient internal loading control. In contrast to the reduction of gRNAs observed with RNAi-mediated repression of mRPN1 in procyclic cells, the population of gRNAs was essentially unchanged after loss of mRPN1 in either mRPN1 conditional null cells or mRPN1 null cells. The detection of larger molecular weight gRNA precursors using guanylyltransferase has been variable in previous reports. While knockdown of KRET1 generated gRNA precursors that were readily detected by guanylyltransferase labeling, no such gRNA precursors were observed after mRPN1 knockdown (Aphasizheva and

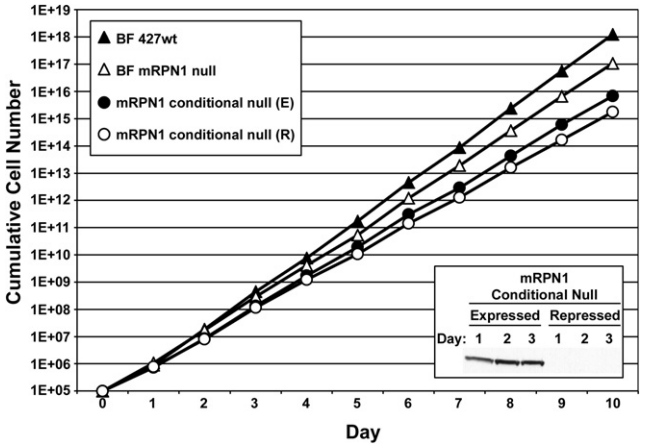

FIGURE 2. Analysis of 427 wt, mRPN1 null, and mRPN1 conditional null cells shows no significant defect in bloodstream form growth in the absence of mRPN1. The graph shows cumulative growth of cells expressing mRPN1 (427 wt and mRPN1 conditional null [E]; solid symbols) compared with cells lacking mRPN1 (mRPN1 null and mRPN1 conditional null [R]; open symbols). The *inset* shows an anti-TAP-tag Western blot of tet-regulated mRPN1 expression in the mRPN1 conditional null cell line, revealing the absence of tagged mRPN1 in the absence of tet.

Aphasizhev 2010; Madina et al. 2011). The reason for this difference is unclear, although Madina et al. (2011) suggest a short half-life and low abundance for gRNA precursors. In our experiments, larger molecular weight bands similar in size to gRNA precursors seen after KRET1 knockdown did appear to be more prevalent in mRPN1 repressed conditional null cells; however, these same bands appear to be essentially equivalent in mRPN1 null cells compared with 427 wt. We are uncertain whether these bands are in fact gRNA precursors. If they are precursors, then this result suggests that immediately following the loss of mRPN1 gRNA precursors accumulate, but in the prolonged absence of mRPN1 cells can reestablish normal gRNA processing via other means. In any case, the persistence of a normal gRNA population after repression of mRPN1 in conditional null cells or the complete absence in mRPN1 null cells demonstrates that mRPN1 is either not involved in gRNA processing in bloodstream form cells, or it plays a role that is insufficient. This result also clearly shows the existence of a gRNA-processing activity whose component(s) remain(s) unidentified.

The effect of the loss of mRPN1 on gRNA polycistron processing, mitochondrial mRNA polycistron processing, and RNA editing in vivo was assessed by quantitative real-time PCR (Fig. 4). Using either β-tubulin or 18S rRNA as an internal reference, the amounts of target RNAs in mRPN1 null cells were determined relative to parental 427 wt cells, while target RNAs in repressed mRPN1 conditional null cell line were determined relative to the same cell line with mRPN1 expressed. As expected, mRPN1 mRNA is not detected in either mRPN1 null cells or repressed mRPN1 conditional null cells, providing corroborating evidence for the elimination of both endogenous mRPN1 alleles in these cell lines. Because RNAi-mediated loss of mRPN1 in procyclic cells led to increased unprocessed gRNA polycistrons, we examined the amounts of unprocessed gRNA polycistrons in bloodstream cells lacking mRPN1 using the same primer sequences used to examine RNAs from procyclic cells in Madina et al. These primers flank the junction between two gRNAs in an unprocessed polycistron, and therefore serve as a proxy for gRNA-processing cleavage activity in vivo. In contrast to the results observed in procyclic cells, loss of mRPN1 in both mRPN1 null and mRPN1 conditional null cells in bloodstream form cells did not alter the relative amounts of unprocessed gRNAs as measured at junctions for gA6-gCYb, gA6-gCR3, gCYb-gCR3, and gCR3-gCYb. The absence of an effect on multiple unprocessed gRNA species shows that mRPN1 is not required for normal gRNA processing in bloodstream form cells, and is consistent with the lack of an effect on the total gRNA population observed in guanylyltransferase labeling assay (Fig. 3). Similar analyses to measure unprocessed mitochondrial mRNAs by examining CYb-A6 and RPS12-ND5 junctions also revealed no changes in repressed mRPN1 conditional null cells, while CYb-A6 appeared to decrease somewhat in mRPN1 null cells. The observed reduction of CYb-A6 unprocessed RNA also appears to mirror a general trend of slightly reduced maxicircle mRNAs including preedited, edited, and never-edited transcripts in mRPN1 null cells compared with 427 wt. This

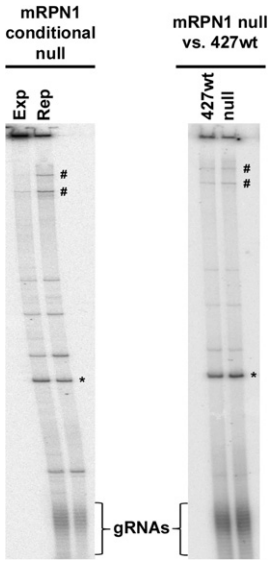

FIGURE 3. Guanylyltransferase labeling shows that gRNAs are retained in the absence of mRPN1 in bloodstream form cells. Guanylyltransferase was used to detect gRNAs within total RNA samples isolated from mRPN1 conditional null cells (*left* panel) with regulated mRPN1 either expressed (Exp) or repressed (Rep), or from 427 wt and mRPN1 null cells (*right* panel). Brackets denote location of mature gRNAs. Asterisk denotes an artifact typically observed in this assay that serves as an internal loading control. Hashtags denote bands consistent with the sizes similar to previously reported gRNA precursors.

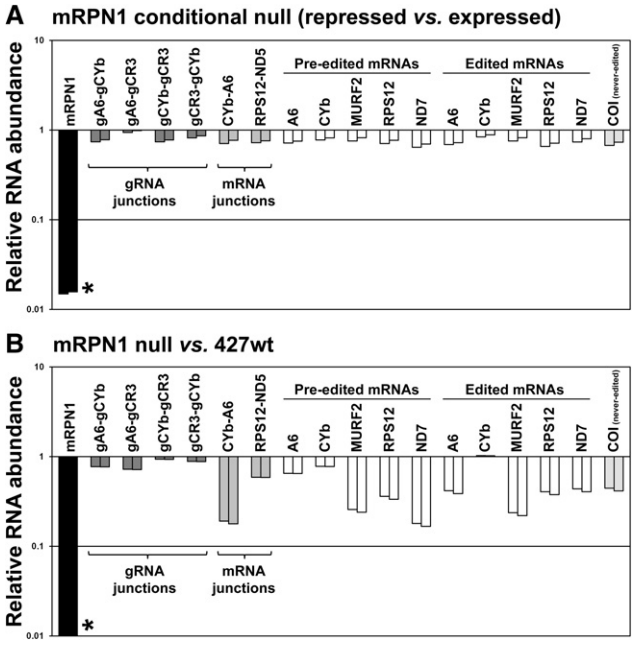

FIGURE 4. Real-time PCR analysis indicates that loss of mRPN1 does not prevent gRNA processing in bloodstream form cells. Relative RNA abundance is shown for nuclear mRNA mRPN1 (black bars), gRNAs junctions (dark gray bars), mRNA junctions (medium gray bars), preedited and edited mRNAs (white bars), and never-edited COI (light gray bars). For each target amplicon, the relative change in RNA abundance was determined by using either 18S rRNA (*left* bar in each pair) or β-tubulin (*right* bar in each pair) as an internal control. (*A*) RNA abundance in cells with mRPN1 repressed calculated relative to cells with mRPN1 expressed shows no change in gRNA processing, mRNA processing, or maxicircle transcript abundance. (*B*) RNA abundance in mRPN1 null cells calculated relative to parental cell line (427 wt) shows no defect in gRNA processing, and minimal losses in maxicircle transcripts including mRNA junctions. Asterisks highlight that mRPN1 mRNA was not detected in mRPN1 null cells or mRPN1 conditional null cells with mRPN1 repressed.

reduction is not observed in mRPN1 conditional null cells (when repressed is compared with expressed) suggesting that this effect is not a consequence of loss of mRPN1. Both preedited and edited transcripts for A6, CYb, MURF2, RPS12, and ND7 are unchanged in repressed mRPN1 conditional null cells, as is never-edited COI. In contrast, RNAi knockdown of mRPN1 in procyclic cells specifically decreased edited A6, CYb, MURF2, RPS12, and ND7 while preedited transcripts remained unchanged (Madina et al. 2011). Thus, while loss of mRPN1 in procyclic form cells led to decreased gRNAs and subsequently a specific reduction in editing, loss of mRPN1 in bloodstream form cells does not alter gRNA abundance and does not substantially alter RNA editing.

The data presented here definitively show that mRPN1 is not essential in bloodstream form *T. brucei* and that its elimination results in no significant defects in either gRNA processing or RNA editing. In contrast, an RNAi knockdown of mRPN1 in procyclic cells reported by Madina et al. showed defective growth, loss of editing and a decrease in mature gRNAs. What is the basis for the difference observed between procyclic and bloodstream form *T. brucei*? The most parsimonious explanation is that the reported RNAi knockdown of mRPN1 in procyclic forms had off-target effects, which can result from sequences with limited similarity (Jackson et al. 2003; Birmingham et al. 2006). Thus, a role for mRPN1 in gRNA processing may have been incorrectly assigned to mRPN1 and the effects on gRNAs and RNA editing may have been secondary to the inhibition of cell growth. Indeed, independent RNAi knockdown of mRPN1 in procyclic cells did not result in defective growth despite target mRNA depletion in two independent cell lines using distinct targeting sequences (R Aphasizhev, pers. comm.) Endogenous expression of mRPN1 is similarly inconsistent with the reported role in gRNA processing. Results from recent ribosome profiling experiments indicate that very little mRPN1 mRNA is actively translated in either procyclic or bloodstream form cells. Published ribosome profiling data reveal that mRPN1 is in the bottom 10th percentile (procyclic) or bottom 18th percentile (bloodstream) among ~8300 transcripts ranked by number of protected RNAs identified (Vasquez et al. 2014). By comparison, known gRNA binding proteins GRBC1/GRBC2 (*aka* GAP1/GAP2; Tb927.7.2570/Tb927.2.3800) are detected with considerable frequency, and are ranked in the top 88th to 93rd percentile in both life cycle stages (Weng et al. 2008; Hashimi et al. 2009). The enzyme that adds U tails to gRNAs, KRET1, was found in the 70th percentile in procyclic cells, and the 55th percentile in bloodstream form cells (Aphasizheva and Aphasizhev 2010). The reported low level of mRPN1 translation appears insufficient for an enzyme responsible for processing gRNAs, which are essential. Additional ribosome profiling data with more extensive and deeper sequencing depth similarly show mRPN1 is in the bottom 16th percentile (procyclic) or bottom 10th percentile (bloodstream) among ~9100 transcripts ranked; GRBC1/GRBC2 were again between 88th and 92nd percentile in both life cycle stages, while KRET1 was found in the 71st and 53rd percentiles in procyclic and bloodstream cells, respectively (Jensen et al. 2014). By way of comparison, ribosome-protected mRNAs above the bottom 16th percentile in this procyclic stage data include VSG pseudogenes (e.g., Tb927.5.4900, Tb927.9.17390, Tb927.10.16470) and JBP1 (Tb927.11.13640), proteins that are expected to have no function in this life cycle stage and are considered to be "silent" (Van Leeuwen et al. 1998; Pays 2005). The ribosome profiling data therefore indicate that mRPN1 expression at the protein level is exceeding low, essentially indistinguishable from biological noise. The sum of existing data strongly suggests that mRPN1 is not involved in gRNA processing in either life cycle stage. However, we cannot exclude alternative explanations that mRPN1 may function differently between life cycle stages, either due to differences in specific gRNA requirements for oxidative phosphorylation in procyclic cells or as a part of the unknown mechanism

regulating developmental changes in RNA editing (Schnaufer et al. 2002).

In summary, we show that mRPN1 is dispensable for growth and gRNA processing in bloodstream form *T. brucei* and also appears to not be essential in procyclic forms. Thus, mRPN1 does not play a critical role in processing of gRNAs that are essential in both life cycle stages. Our results therefore demonstrate that an undiscovered source of gRNA processing must exist, adding yet another layer to the increasingly complex RNA metabolism of trypanosomes.

MATERIALS AND METHODS

Plasmid constructs

Plasmids pSKO-mRPN1 (single knockout) and pDKO-mRPN1 (double knockout) were created using published methods to eliminate both *mRPN1* alleles in BF 427 wild-type cells (Wirtz et al. 1999; Schnaufer et al. 2001). To generate pSKO-mRPN1, the UTRs of *mRPN1* were PCR amplified from 427 genomic DNA using primers 5FOR-8178 (ATAGCGGCCGCGGAATAAATAGGAGTGCT AACAAG) and 5REV-8179 (ATAACGCGTCTCGAGACTGCCC GGATTACACTCTG) or 3FOR-8180 (ATATCTAGAATTTAAATC TTTTCCGAGCGAGGGAACAA) and 3REV-8181 (ATAAGGCC TGCGGCCGCATTCACTCCTTTCCTCCACGTTG). The 366-bp *mRPN1* 5′ UTR and 329-bp *mRPN1* 3′ UTR PCR products were cloned into the NotI/MluI and StuI/XbaI sites of pLEW13, respectively, creating pSKO-mRPN1. pDKO-mRPN1 was created by replacing the SwaI/XhoI fragment (containing Neo^r marker) of pSKO-mRPN1 with the 2491-bp StuI/XhoI fragment (containing Hyg^r marker) from pLEW90.

The wild-type *mRPN1* gene was cloned into the pLEW-MHTAP plasmid (Jensen et al. 2007), creating the pReg-mRPN1 plasmid as follows. A 1460-bp PCR product containing the *mRPN1* open reading frame (minus the last 5 codons) was PCR amplified from 427 genomic DNA with Fidelitaq polymerase (Affymetrix) using 5orf-8171 (ATAAAGCTTATGATTCGCTTGAGCGAAG) and 3orf-8173 (ATAGGATCCGGAAGGCGAAGCAAACCATAG) primers. This *mRPN1* ORF was cloned into pGEM-T Easy plasmid (Promega). Site-directed mutagenesis (Stratagene) was performed using primers FOR-8174 (CTGCCGTGTATCATGGTGACCCCGCAAC ACTTTGGA) and REV-8175 (TCCAAAGTGTTGCGGGGTCACC ATGATACACGGCAG) to remove an internal BamHI site. The HindIII/BamHI *mRPN1* ORF was subcloned into the "same" sites in pLEW-MHTAP, and site-directed mutagenesis with primers 5fix-8358 (GTTTGCTTCGCCTTCCCGGATCCATGGAACAGA) and 3fix-8359 (TCTGTTCCATGGATCCGGGAAGGCGAAGC AAAC) was done to place the *mRPN1* sequence in frame with the TAP-tag in pLEW-MHTAP. This plasmid was used to create cell lines with tetracycline-induced expression of an ectopic, TAP-tagged *mRPN1* allele from the *rDNA* locus.

Cell lines

The mRPN1 null was generated by sequential transfections to introduce the pSKO-mRPN1 and pDKO-mRPN1 plasmids sequentially into 427 strain cells. First, cells were transfected with 10-μg NotI-linearized pSKO-mRPN1, and recombinants were selected by G418 resistance. The second endogenous *mRPN1* allele was eliminated by transfection with 10-μg NotI-linearized pDKO-mRPN1 and subsequent hygromycin selection. Cells were grown in HMI-9 media containing 2.5 μg/mL G418 and 5 μg/mL hygromycin. A regulatable ectopic *mRPN1-TAP* allele was introduced into the *rDNA* intergenic locus by transfection with NotI-linearized pReg-mRPN1 and selection in 2.5 μg/mL phleomycin, generating mRPN1 conditional null cell line. Induction of pReg-mRPN1 used 1 μg/mL tetracycline.

PCR analysis of genomic DNA

PCR was used to determine if the *mRPN1* (Tb927.11.8400) coding sequence had been eliminated by the intended homologous recombinations. Genomic DNA was isolated using DNeasy Blood & Tissue Kit spin columns (Qiagen). Four sets of primers were used to detect all or parts of the *mRPN1* coding sequence: Primers FOR-8217 (CACTCATTTGTTTATTCATTTGTTTCGG) and REV-8218 (CTTCCCCCATAATATTTTGTTCCCTCG) amplify nucleotides −38 to 1526 (positions relative to ATG; 1564-bp product), primers FOR-8217 and REV-8261 (TCCTCAGTAATGGCATG AAGTGAT) amplify nucleotides −38 to 437 (475-bp product), primers FOR-8260 (CCAGAAGGTGGGAGGGAATAC) and REV-8218 amplify nucleotides 362–1526 (1165-bp product), and primers FOR-8260 and REV-8261 amplify nucelotides 362-437 (76-bp product). Primers that flank the junction between the inserted knockout construct and the genomic regions 5′ and 3′ of *mRPN1* were used to amplify products that demonstrate the intended homologous recombinations. Primers 5int-8403 (GGTG TGCACACTTCACAAAAC) and pLEW13rev5153 (CTGATAGC TAAGCTATCGCA) generate a product of 526 bp, and primers pLEW13for5154 (CATTGCAGTCTCCGCTCTTA) and 3int-8404 (GGCAGTTGTGACATTGTGTTG) generate a product of 454 bp; these indicate integration at the 5′ and 3′ ends of the first knockout construct, respectively. Primers 5int-8403 and pLEW90rev5208 (GCAGCTCTAATGCGCTGTTA) generate a product of 702 bp, and primers pLEW90for5209 (GGAATAGAGTAGATGCCGAC) and 3int-8404 generate a product of 682 bp; these indicate integration at the 5′ and 3′ ends of the second knockout construct, respectively.

Growth of cells in vitro

BF cells were grown in HMI-9 with 10% FBS. Cell density was measured by Coulter counter, with cultures reseeded at 2×10^5 cells/mL daily.

Western analysis

Tetracycline-regulated expression of TAP-tagged mRPN1 was monitored by Western blot. For cells grown with or without tetracycline, equivalent cell numbers were harvested (4.5×10^6 cells) and resuspended in 40-μL SDS load dye. Twenty microliters of each sample was resolved on Criterion 10% SDS-PAGE gel (BioRad), transferred to Immobilon-P membrane (Millipore), and blocked overnight at 4° C in 1× PBS-T with 5% milk. Blots were probed with recombinant peroxidase antiperoxidase (Sigma) at 1:2000 and subsequently washed four times with 1× PBS-T and developed with ECL kit (Pierce) per manufacturer's instructions.

Guanylytransferase labeling of gRNAs

Total RNA was extracted from cells using TRIzol (Life Technologies) following manufacturer's instructions. RNA samples were DNase I treated using the DNA-free kit (Ambion) following manufacturer's instructions. 0.9 µg of RNA was incubated at 37°C for 1 h in a 15 µL reaction containing 40 µCi [α-32P]GTP (3000 Ci/mmol) and 10 units of guanylyltransferase (Epicentre Biotechnologies) following manufacturer's instructions. The reaction products were separated by electrophoresis on 9% polyacrylamide gel containing 7 M urea and 1× TBE and subsequently visualized by PhosphorImager (GE Healthcare).

Real-time PCR analysis

Real-time PCR was performed as previously described, with values normalized to either 18S rRNA or β-tubulin and an internal control (Carnes et al. 2005, 2008; Ernst et al. 2009). Primers to detect mRPN1 as well as gRNA junctions A6-CYb, A6-CR3, CYb-CR3, and CR3-CYb were previously described (Madina et al. 2011). For each RNA measured, an average quantification cycle (C_Q) value was used for calculations. Relative changes in target amplicons were determined by using the Pfaffl method, with PCR efficiencies calculated by linear regression using LinRegPCR (Pfaffl 2001; Ramakers et al. 2003).

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