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Biphasic decay of guide RNAs in *Trypanosoma brucei*

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Abstract

Guide RNAs (gRNAs) are short mitochondrially encoded RNAs that contain the information for editing of messenger RNAs in *Trypanosoma brucei*. Although a great deal of work has focused on the utilization of gRNAs in editing, little is known about the turnover of gRNAs. In this report, we utilized in organello pulse chase and in vitro RNA decay experiments to directly examine gRNA turnover. We found that gRNAs are degraded by a biphasic mechanism. In the first step of decay, 3′ gRNA sequences encompassing primarily the post-transcriptionally added oligo(U) tail are rapidly removed. This is followed by a second step, which entails a comparatively slower degradation of the encoded gRNA body. Decay of the 3′ end of the gRNA is sequence specific, as it does not occur on oligoadenylated gRNAs. In contrast, the nucleotide composition of the 3′ extension does not affect the rate of degradation during the second, slower, decay step. Finally, competition assays suggest that complete gRNA decay is mediated by two distinct enzymes, one of which simultaneously recognizes elements of the oligo(U) tail and the encoded portion of the gRNA. Overall, these results provide the first evidence for a gRNA-specific decay pathway.

Keywords: RNA decay; RNA turnover; RNA editing; Mitochondrial RNA

1. Introduction

The expression of 12 out of 18 mRNAs in the mitochondria of *Trypanosoma brucei* requires a post-transcriptional processing event known as RNA editing. RNA editing involves the precise insertion and deletion of uridine residues in mRNAs, by which it creates open reading frames for otherwise untranslatable RNAs [1–3]. A critical component in this process is a group of small (50–70 nt) mitochondrially encoded RNAs called guide RNAs (gRNAs). gRNAs were first identified by Blum and colleagues as small RNA molecules with sequences that were complementary to edited regions of mature mRNAs, leading to the model that editing of mRNA is directed by information encoded on gRNAs [4]. Seiwert and Stuart experimentally confirmed the function of gRNAs by demonstrating that the number inserted or deleted uridines could be directed by gRNA sequence changes in in vitro editing assays [5]. Despite their sequence heterogeneity, gRNAs can be divided into three functional domains [5–9]. The 5′ most region of the gRNA, the anchor region, is complementary to the mRNA just 3′ of the sequences whose editing it directs. The binding of the anchor region to the corresponding mRNA is thought to initiate the editing reaction by directing nucleolytic attack 5′ of the gRNA/mRNA duplex (relative to the mRNA) [6]. The middle of the gRNA contains the guiding or information region, which encodes the sequences necessary to ensure proper editing of the mRNA. This guiding region is complementary to the edited RNA and specifies the edited sequence through Watson Crick and G:U basepairing interactions [5]. The 3′ end of the gRNA is composed of a postranscriptionally added oligo(U) tail, which is 5–24 nucleotides long with an average length of 15 nucleotides [7]. The oligo(U) tail is thought to stabilize the interaction between gRNAs and mRNAs through base pairing [8]. gRNAs not only contain similar functional domains, but possess a common secondary structure of an imperfect double stem loop [9].

The abundance of gRNAs has been shown to vary between different life cycle stages [10–12]. For the small number of gRNAs that have been examined, their abundance does not vary in parallel with the developmentally regulated accumulation of the cognate edited mRNA [10,11]. Thus, it has been concluded that gRNA abundance does not control life-cycle stage specific

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editing. In fact, in some cases, developmental accumulation of specific gRNAs is the inverse of the levels of the corresponding edited mRNA, suggesting that gRNAs may be degraded as a consequence of the editing process [11,12]. On the other hand, we recently demonstrated that down-regulation of gCYb[560] correlates closely with a decrease in CYb mRNA editing [13]. These results suggest that the abundance of some gRNAs may be limiting, and that there could be specific instances in which the level of a given gRNA controls the occurrence of the corresponding editing events.

While the structure and function of gRNA molecules are well defined, the mechanisms that govern gRNA expression are relatively poorly understood. gRNA abundance could be regulated at the levels of transcription, processing and/or RNA turnover. With few exceptions, gRNAs are encoded on small DNA molecules termed minicircles [14,15]. The current model of transcription suggests that initiation occurs just upstream of each gRNA regardless of its location on the minicircle [16,17]. Whether different gRNA promoters are differentially regulated is unknown. Transcription can proceed through downstream gRNAs, resulting in polycistronic primary transcripts [16]. However, in vitro studies suggest that only the 5′ most gRNA in a polycistronic transcript will be processed into a mature gRNA through ribonuclease and terminal uridylyl transferase (TUTase) action [18]. Recent knockdown experiments demonstrated that RNA Editing TUTase 1 (RET1) is responsible for at least some fraction of oligouridylation of gRNAs in vivo [19]. Regarding the mechanisms of gRNA turnover, little is known. The only in vitro study to address this subject showed that addition of the oligo(U) tail is both stimulated and stabilized by the presence of the corresponding cognate mRNA [18]. In the absence of evidence for transcriptional regulation of specific gRNA genes, it remains possible that gRNA turnover plays a role in regulating gRNA levels both in conjunction with and/or prior to usage in editing.

Here, we report the first evidence for a gRNA-specific turnover pathway, and describe the biphasic mechanism of this decay. Utilizing in organello pulse labeling experiments we find that the majority of the gRNA label is lost in less than a minute, much more rapidly than oligouridylated rRNAs. Subsequent in vitro turnover experiments using mitochondrial extracts reveal that decay of gRNAs occurs in two steps. First, the 3′ oligo(U) tail and a few encoded nucleotides are rapidly removed. In the second step, the gRNA body is slowly degraded. We show that an oligo(U) tail as short as 5 nucleotides can support the initial decay step, although shortening the tail affects the size of stable degradation intermediates. The initial rapid decay phase is sequence specific, as it does not take place on gRNAs with an oligo(A) tail. However, the rate of decay of the encoded portion of the gRNA is independent of the 3′ homopolymer sequence. Finally, competition experiments suggest the involvement of two different ribonucleases, one of which displays a high affinity for polyuridylated gRNA. Together, our results indicate the presence of a rapid gRNA decay pathway that requires the combination of both the oligo(U) tail and the encoded portion of the gRNA.

2. Materials and methods

2.1. Cell culture, mitochondrial isolation, and mitochondrial extract preparation

Procyclic form *T. brucei brucei* clone IsTaR1 stock EATRO 164 (Figs. 1–5) or strain 427 (Fig. 6) were grown as previously described [20]. Mitochondrial vesicles were isolated on linear 20–35% Percoll gradients and stored at −80 °C [21]. Mitochondrial extracts were prepared as previously described [22]. In this report only the salt-extracted mitochondrial membrane protein (EMP) fraction was utilized.

2.2. Post-transcriptional in organello labeling assays

In organello pulse chase assays were performed essentially as described with minor modifications [23,24]. Following collection, mitochondrial vesicles were resuspended at a concentration of 5 mg/ml in transcription buffer (5 mM HEPES (pH 7.6), 3 mM potassium phosphate (pH 7.8), 125 mM sucrose, 6 mM KCl, 10 mM $MgCl_2$, 1mM EDTA, 2 mM 2-mercaptoethanol) containing 0.1 mM ATP. Vesicles were labeled with [α^{32}P]UTP (800 Ci/mmol) at a concentration of 200 μCi/ml for 10 min at 27 °C in the absence of the remaining three NTPs to measure post-transcriptional UTP incorporation. Excess label was removed and vesicles were then resuspended in transcription buffer containing 2 mM UTP and incubated at 27 °C for times indicated. Pulse reactions were carried out in a volume of 50 μl, and chase reactions were performed in batch with a volume of 50 μl per time point. At indicated time points, 50 μl was removed from batch chase reactions for termination. Reactions were stopped as described, total RNA was isolated, and equal volumes of each reaction were analyzed by electrophoresis on a 6% acrylamide/7 M urea gel. Gels were analyzed either by autoradiography followed by densitometry of non-saturated autoradiographs using a Bio-Rad GS-700 imagining densitometer and Multi-Analyst software (version 1.1) or by phosphoimager analysis on a Bio-Rad Personal FX Phosphoimager using Quantity One software (Version 4.2.1).

2.3. Oligodeoxynucleotides

The following oligodeoxynucleotides were used in these studies (Integrated DNA technologies). T7 promoter sequences are underlined.

RPS12u-5′T7, (5′-TGTAATACGACTCACTATAGGGCTAATACACTTTTGATAACAAACTAAAGTAAA-3′); RPS12u (5′-AAAAACATATCTTATTCT-3′); RPS12u-A20 (5′-TTTTTTTTTTTTTTTTTTTTAAAAACATATCTTATTCT-3′); gA6[14]-5′T7 (5′-TGTAATACGACTCACTATAGGGGGGCGAATTCATATATAC-3′); gA6[14]-NT (5′-TAATTATCATATCACTGTCAA-3′); gA6[14]-5U (5′-AAAAATAATTATCATATCACTGTCAA-3′); gA6[14]-10U (5′-AAAAAAAAAATAATTATCATATCACTGT CAA-3′); gA6[14]-17A (5′-TTTTTTTTTTTTTTTAATTATCATATCACTGTC AA-3′).

2.4. RNA synthesis

RPS12un and RPS12un-A20 RNAs were synthesized as previously described [22]. The guide RNA gA6[14]-17U was synthesized directly from a previously described plasmid [25]. This plasmid was also used as a template to generate PCR products for creation of the remaining RNAs used in this study. This was achieved by amplifying the plasmid with the sense oligonucleotide gA6[14]-5′T7 and the antisense primers gA6[14]-NT, gA6[14]-5U, gA6[14]-10U or gA6[14]-17A. These PCR products were utilized to generate gA6[14] with no 3′ extension, a 5 uridine extension, 10 uridine extension, or 22 adenosine extension. The gA6[14]-22A transcript was synthesized from a PCR product created using the gA6[14]-17A oligonucleotide. However, we consistently observed that the transcript from this template appeared larger than expected, and DNA sequencing showed that PCR amplification generated a product with a 22 nucleotide poly(A) tail rather than a 17 nucleotide poly(A) tail. This difference is reflected in the length of the resulting RNA (Fig. 4). The guide RNAs gCYb[558]-15U and gCYb[558] were synthesized directly from a previously described plasmid [25]. RNAs were synthesized with T7 RNA polymerase and [α^{32}P]GTP from the templates described above using the Megascript in vitro transcription system (Ambion Inc.). Labeled RNAs were separated on a 6% acrylamide/7 M urea gels and visualized by UV shadowing. Full-length products were excised, eluted from the gel, and concentrated by isopropanol precipitation.

2.5. RNA degradation assays

RNA degradation assays were performed as previously described, up to and including termination of the reaction [22]. Following termination 5 μl stop buffer (0.2% SDS and 50 mM EDTA) was added and RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). Twenty microlitres of the aqueous phase was then added to 20 μl of 90% formamide loading buffer and heated at 90 °C for 3 min. Equal volumes of each fraction were analyzed by electrophoresis on 6% acrylamide/7 M urea gels and autoradiography. Non-saturated autoradiographs were analyzed using a Bio-Rad GS-700 imaging densitometer and Multi-Analyst software (Version 1.1) or gels were exposed to phosphoimager screen and analyzed with a Bio-Rad Personal FX Phosphoimager and Quantity One software (Version 4.2.1). Percent full-length RNA remaining was determined by analyzing density at the migration position of the starting material. Percent total RNA was determined by analyzing density of the area between and including full-length and intermediate product RNAs as described in the text. Half lives are expressed as the mean ± standard deviation of three experiments.

In ribohomopolymer competition experiments, unlabeled poly(U) (Amersham Biosciences), poly(C) (Sigma), or yeast tRNA (Sigma) was added in different molar ratios (relative to labeled gRNA) to each reaction. The moles of competitor RNA were approximated from the average length as determined from the specifications sheet.

Because ribonuclease activity varies between preparations, each EMP preparation was initially characterized by protein titration. Following protein titration, the protein concentration that exhibited optimal poly(A) specific RNA degradation activity [22] was used in each reaction.

3. Results

3.1. Guide RNAs are rapidly degraded in isolated mitochondria

To gain insight into the mechanisms of gRNA turnover, we began by analyzing the process in organello. Isolated mitochondria were labeled with [α^{32}P]-UTP for 10 min in the absence of other nucleotides to prevent transcription [21,23]. Previous work has demonstrated through RNA sequencing [26] and gel electrophoresis and hybridization [27] that under these conditions, [α^{32}P]-UTP is incorporated post-transcriptionally into 9S rRNA, 12S rRNA and gRNAs, apparently through labeling of their 3′ oligo(U) tails. To measure the relative stabilities of rRNAs and gRNAs, isolated mitochondria were pulsed with [α^{32}P]-UTP and chased with 2 mM unlabeled UTP for up to 30 min. RNA was isolated from the mitochondria, resolved on 6% acrylamide/7 M urea gels, and visualized by autoradiography. Under these conditions, turnover of the labeled gRNA

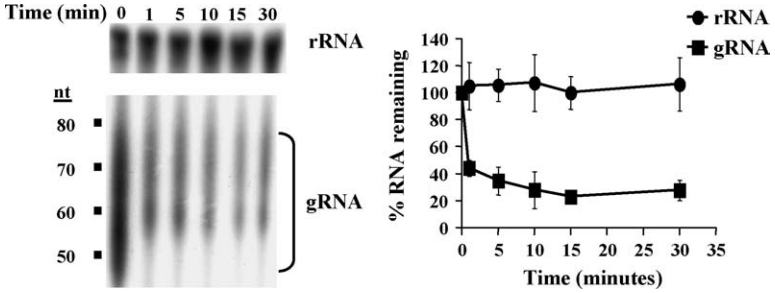

Fig. 1. In organello turnover of mitochondrial gRNAs and rRNAs. Mitochondrial vesicles were labeled with [α^{32}P]-UTP for 10 min. Excess label was removed and vesicles were resuspended and incubated for up to 30 min in 2 mM unlabeled UTP. RNAs were isolated and analyzed on a 7 M urea and 6% acrylamide gel and visualized by autoradiography in the left panel. RNA size standards are indicated on the left (nt, nucleotides) and rRNA and gRNA populations are designated on the right. Percent RNA remaining is graphed in the right panel. 9S and 12S rRNAs are graphed as a single population because they were not well resolved in all experiments. Error bars represent one standard deviation obtained from three experiments.

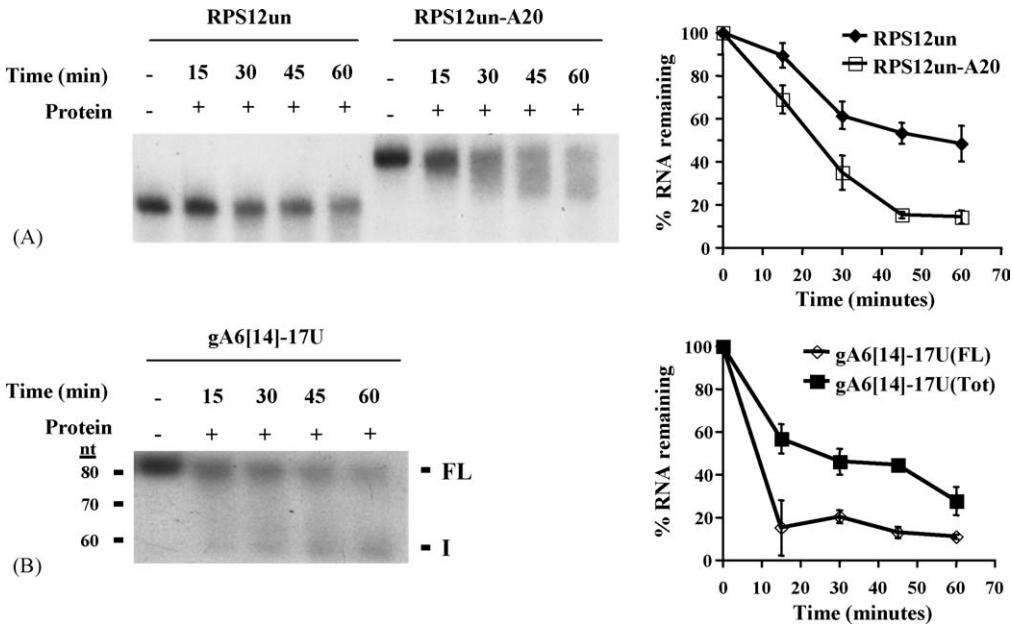

Fig. 2. Rapid decay of the gRNA gA6[14]-17U by partially purified mitochondrial protein extract. In vitro transcribed and internally labeled RPS12un and RPS12un-A20 (A) or gA6[14]-17U RNAs (B) (2.5 pmol each) were incubated separately with partially purified mitochondrial extract at 27 °C for the indicated times. Products were resolved on a 7 M urea and 6% acrylamide gel and visualized by autoradiography in the left panels. The (–) Protein and (–) Time (min) lanes indicate starting material. Migration positions of full-length (FL) and intermediate products (I) for gA6[14]-17U are designated to the right. Percent RNA remaining was determined by densitometry and is plotted for each time point in the right panels. Error bars represent one standard deviation obtained from three independent experiments.

population was very rapid. Less than 50% of labeled gRNA remained after 1 min of incubation with unlabeled UTP (Fig. 1). In contrast, both 9S and 12S rRNAs were very stable. Little or no decay of either rRNA was observed even after 30 min of chase with unlabeled UTP (Fig. 1). These results demonstrate that, in organello, full-length gRNAs are rapidly degraded with a half life of less than 1 min. However, since labeling of gRNA in this system occurs by incorporation of [α^{32}P]-UTP into non-

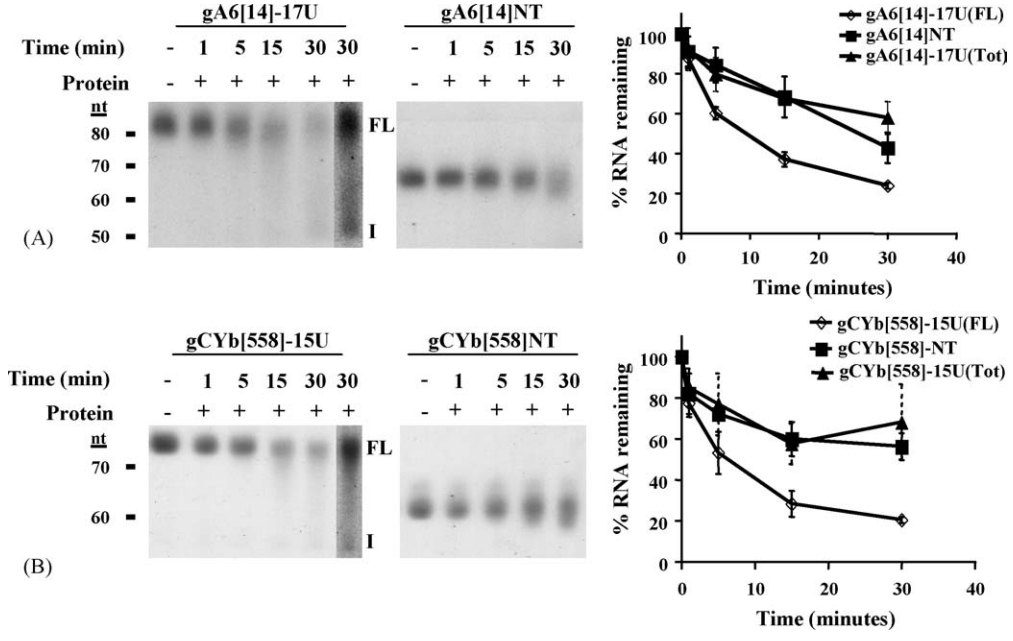

Fig. 3. Biphasic decay of gRNAs in vitro. gA6[14] (A) and gCYb[558] (B) with or without an oligo(U) tail were internally labeled. These RNAs were incubated with partially purified mitochondrial extract at 27 °C for the indicated times. Products were resolved on 7 M urea and 6% acrylamide gels and visualized by autoradiography in left panels. The (–) Protein and (–) Time (min) lanes indicate starting material. Migration positions of full-length (FL) and intermediate products (I) for polyuridylated RNAs are designated to the right of those panels. To enhance visualization of intermediate products, a duplicate lane of the 30 min degradation of oligouridylated RNAs is shown, after darker exposure, to the right of those experiments. Percent RNA remaining was determined by densitometry and is plotted for each time point in the right panels. Error bars represent one standard deviation obtained from three independent experiments. Comparison of half lives using a student's *t*-test, shows that the half life of gA6[14]-17U(FL) is different than the half life of either gA6[14]-17U(Tot) or gA6[14]NT ($p < 0.01$). However, gA6[14]-17U(Tot) and gA6[14]NT have statistically similar half lives ($p > 0.10$). Statistical analysis of gCYb[558] degradation provides similar conclusions.

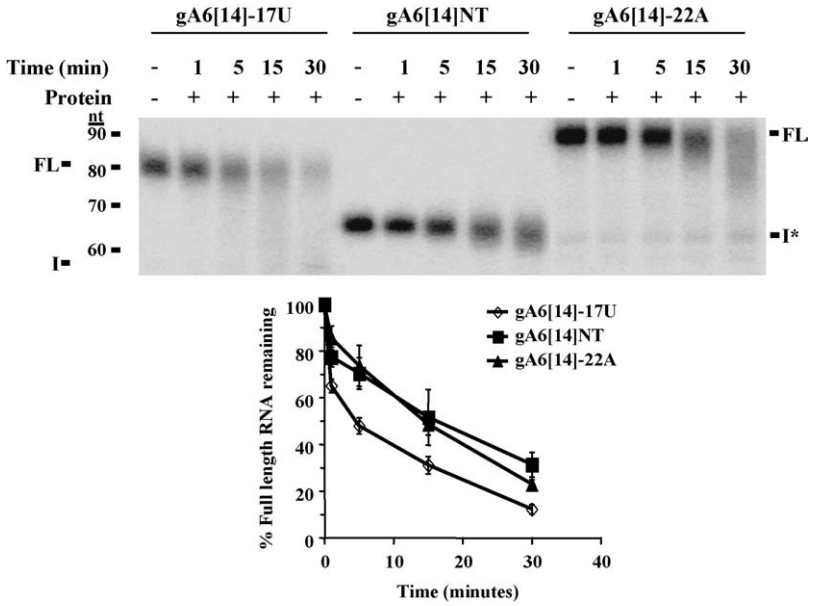

Fig. 4. The initial rapid decay step of gRNA turnover is oligo(U) specific. In vitro transcribed and internally labeled gA6[14]-17U, gA6[14]NT and gA6[14]-22A were incubated with partially purified mitochondrial extract at 27 °C for the times indicated. Products were resolved on a 7 M urea and 6% acrylamide gel and visualized with a phosphorimager screen in top panel. The (–) Protein and (–) Time (min) lanes indicate starting materials. Migration positions of full-length (FL) and intermediate products of gA6[14]-17U decay are indicated to the left, and positions of full-length (FL) and intermediate products (I^*) of gA6[14]-22A degradation are indicated on the right. Percent full-length RNA remaining was determined using densitometry and plotted for each time point in bottom panel. Comparison of the half life of gA6[14]-17U to the half life of either gA6[14]NT or gA6[14]-22A, using a student's *t*-test, shows these values are statistically different ($p < 0.05$).

encoded oligo(U) tail, we cannot distinguish whether the rapid loss of signal we observe is due to deuridylation of gRNAs or to decay of the entire molecule. Regardless of whether the observed loss of gRNA results from deuridylation or total decay of the molecule, the mechanism appears to be specific for gRNA since rRNAs have a similar 3′ end and remain stable throughout the experiment. Alternatively, the entire rRNA population may be rapidly bound by protein and sequestered from the degradation machinery.

In these experiments, we also observed that approximately 30% of the gRNA population remains stable after 30 min (Fig. 1). Furthermore, approximately 60% of the gRNA is degraded within the first minute of the chase period while only 10–15% more of the gRNA degraded over the following 29 min (Fig. 1).

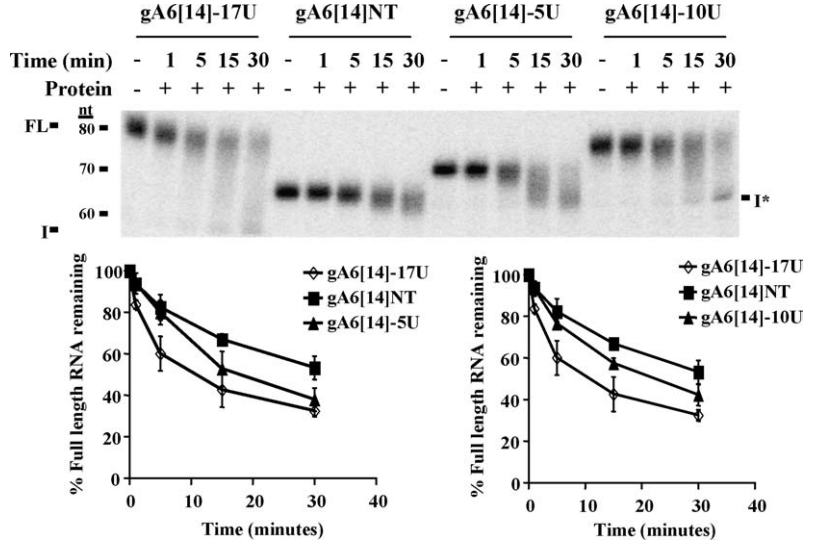

Fig. 5. Effect of oligo(U) tail length on the initial decay step of gRNA turnover. Internally labeled gA6[14] without a tail (gA6[14]NT) or with a 5-, 10- or 17-uridine tail (gA6[14]-5U, gA6[14]-10U, gA6[14]-17U, respectively) were incubated with partially purified mitochondrial extract at 27 °C for the indicated times. Products were resolved on a 7 M urea and 6% acrylamide gel and visualized utilizing a phophorimager screen top panel. The (–) Protein and (–) Time (min) lanes indicate starting materials. The positions of full-length (FL) and intermediate (I) products for gA6[14]-17U are indicated to the left. The intermediate (I^*) products for gA6[14]-5U and gA6[14]-10U are marked on the right. The data were analyzed by densitometry and percent full-length RNA remaining is plotted in the bottom panel. Error bars represent one standard deviation obtained from three experiments.

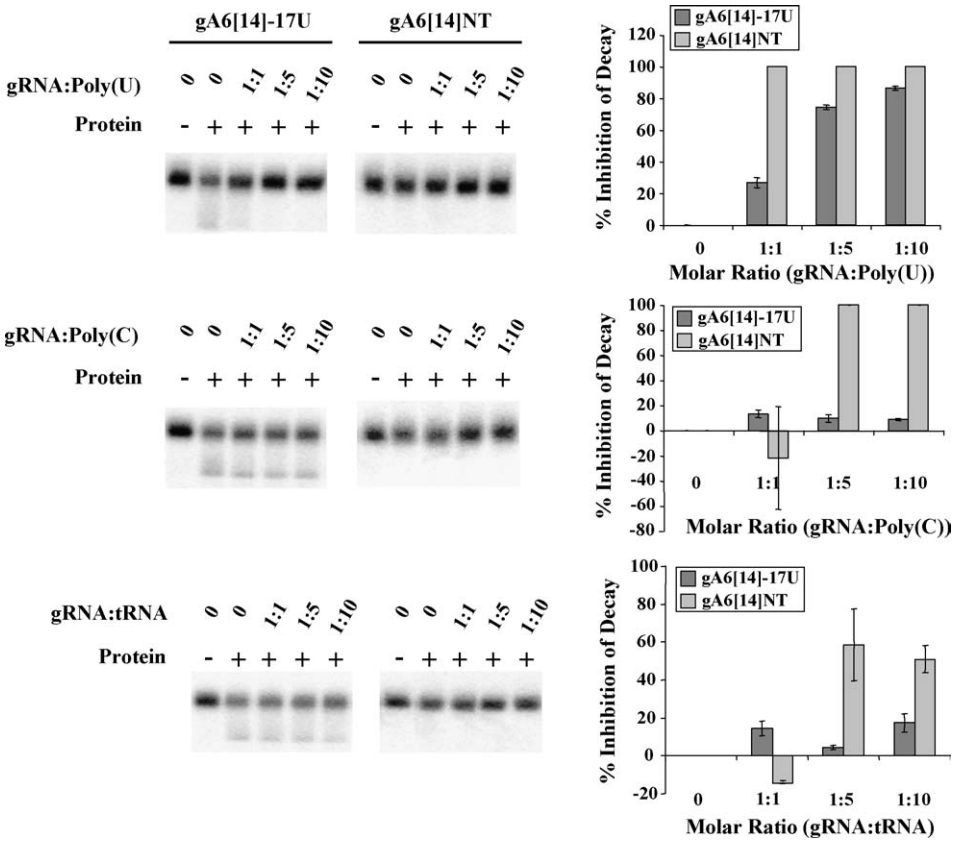

Fig. 6. Decay of the oligo(U) tail and gRNA body are mediated by distinct enzymes. In vitro transcribed and internally labeled gA6[14]-17U or gA6[14]NT (2.5 pmol of each) were incubated separately with the indicated mole: mole ratio of unlabeled poly(U), poly(C), or tRNA for 30 min at 27 °C with partially purified mitochondrial extract. Products were resolved on a 7 M urea and 6% acrylamide gel and visualized utilizing a phosphorimager screen (left panels). gRNAs bearing or lacking oligo(U) tails (gA6[14]-17U and gA6[14]NT, respectively) were aligned for ease of comparison. The (0) gRNA:competitor RNA and (–) Protein lanes indicate starting materials. Percent full-length RNA decay was determined by densitometry. Using these percentages, percent inhibition of decay was calculated as [(percent decay of full length RNA in the absence of competitor – percent decay of full length RNA in the presence of competitor)/percent decay of full length RNA in the absence of competitor]. Duplicate experiments are graphed with error bars indicating the range. In samples in which quantitation indicated greater signal in lanes with competitor compared to lanes with no competitor, the value was set at 100% inhibition.

These results suggest that a portion of the gRNA population is protected from rapid degradation. Furthermore, this average size of this protected subset increases relative to the initial labeling, suggesting that protected gRNAs may still contain an oligo(U) tail. These protected gRNAs could be secluded from nucleolytic attack by binding to the editing machinery, forming a gRNA/mRNA complex, or by interacting with a combination of protein and mRNA factors [1–3,8,18,19,25].

3.2. Decay of gRNAs is biphasic in vitro

Due to the limits of the in organello post-transcriptional labeling system, we were unable to conclude if the rapid loss of gRNA we observe is caused by degradation of the entire gRNA molecule or removal of the labeled oligo(U) tail. Therefore, we utilized a previously established in vitro RNA turnover assay to further examine the mechanisms of gRNA degradation [22]. In this assay, in vitro transcribed body-labeled RNAs were incubated with salt extracted mitochondrial membrane proteins (EMP). RNA degradation was monitored by gel electrophoresis followed by autoradiography or phosphoimager analysis. In these experiments, we utilized derivatives of gA6[14] and gCYb[558], which specify editing of ATPase 6 and apocytochrome B RNA, respectively [10,11].

To analyze the rate of gRNA decay, we first compared the degradation of gA6[14]-17U with that of adenylated and non-adenylated unedited mRNAs (RPS12un-A20 and RPS12un, respectively). Previously, we demonstrated that polyadenylated unedited RNAs are preferentially degraded compared to their non-adenylated counterparts in this system [22]. In these experiments, we initially measured the disappearance of the signal for full-length gA6[14]-17U, regardless of the nature of the decay intermediates. Time course experiments comparing turnover of RPS12un, RPS12un-A20 and gA6[14]-17U showed that the disappearance of full-length gA6[14]-17U (gA6[14]-17U(FL)) was more rapid than that of even polyadenylated RPS12un (compare Fig. 2A and B). The average half lives of RPS12un and RPS12un-A20 were 62.3 ± 9.3 and 23.6 ± 1.8 min, respectively (Fig. 2A). In contrast, gA6[14]-17U(FL) displayed a half life less than 15 min (Fig. 2B). Similar results were observed if micrococcal nuclease-treated extracts were utilized, thereby ruling out any potential effects of cognate mRNAs on the rate of gRNA decay in this system (data not shown). In a previous study, we showed that RPS12un with a 20 uridine tail degrades at the same rate as

RPS12un without an extension [22]. Taken together, these data imply that degradation of gA6[14]-17U occurs by a mechanism different than the mechanism that degrades unedited RNAs in our in vitro system.

In addition to the rapid disappearance of the full-length gRNA, we also observed the formation of an intermediate product (I, Fig. 2B) and a faint smear between the full-length and intermediate bands. Analysis of the migration of these products compared to a 10 base pair ladder showed that the prominent stable intermediates lack the entire U tail and 2–4 nucleotides of the 3′ end of the encoded gA6[14] sequences. These 2–4 nucleotides comprise part of the second stem of gA6[14]-17U. To examine decay of the entire gA6[14]-17U molecule, we quantitated all products between and including full-length RNA and the intermediate product (gA6[14]-17U(Tot); Fig. 2B). This RNA population decayed with an average half life of 27.3 ± 8.5 min, which is similar to the half life of RPS12un-A20. Comparison of the decay of full-length gA6[14]-17U with disappearance of the total gA6[14]-17U population suggests that two steps are involved in the degradation of gRNA in vitro. The first step rapidly removes the 3′ oligo(U) tail and a few nucleotides of encoded 3′ sequence, while a slower second step degrades the bulk of the gRNA body. These results suggest that the rapid disappearance of gRNA observed in Fig. 1 is likely the result of rapid deuridylation.

Next, we asked whether the 3′ oligo(U) extension is required for degradation of gRNA in this system. We compared decay of gA6[14] with and without a 17U tail over a time course (gA6[14]-17U and gA6[14]NT, respectively (Fig. 3A)). The average half life of full-length gA6[14]-17U ((gA6[14]-17U(FL)); 9.4 ± 1.4 min) was 2.7-fold shorter than that of gA6[14]NT (25.5 ± 4.9 min) (Fig. 3A). As described above, we also observed the formation of intermediates consisting of a smear and a relatively stable product upon degradation of gA6[14]-17U (Fig. 3A darker exposure). In the experiments presented in Fig. 3A, the total gA6[14]-17U population (gA6[14]-17U(Tot)) degraded with an average half life of 36.4 ± 7.4 min, which is not statistically different from the half life of gA6[14]NT. These results show that decay of gA6[14]-17U occurs in a two step process in which degradation of the gRNA body appears rate limiting. However, the slower decay of the gRNA body does not require the presence of a oligo(U) tail for activation.

To determine whether this two-step decay process was specific to gA6[14] or if it is common to other gRNAs, we analyzed turnover of gCYb[558] with and without a 15U tail, gCYb[558]-15U and gCYb[558]NT, respectively (Fig. 3B). Time course experiments performed with gCYb[558]-15U and gCYb[558]NT also revealed a biphasic mechanism for decay (Fig. 3B). Full-length gCYb[558]-15U degrades with an average half life of 6.9 ± 2.5 min, which is 5-fold faster than gCYb[558]NT (average half life of 34.8 ± 5.5 min). In addition, degradation of gCYb[558]-15U also produced a smear and an intermediate product that migrates slightly faster than non-uridylated gCYb[558] (I, Fig. 3B). When we quantified decay of the total gCYb[558] population, including stable intermediate and the smear in between (gCYb[558]-15U(Tot)), we observed an average half life of 33.3 ± 12.1 min, a rate of decay that is similar to that of gCYb[558]NT (Fig. 3B). These results demonstrate that regardless of their encoded sequence, oligouridylated gRNAs are degraded by a similar process involving a relatively rapid deuridylation followed by a slower decay of the gRNA body.

3.3. Role of 3′ tail sequence and length in gRNA decay

We next determined the role of 3′ tail sequence in the initial rapid decay event of gA6[14] RNA. We generated gA6[14] transcripts with either no 3′ extension, a 17-uridine extension, or a 22-adenosine extension. These RNAs were incubated separately in in vitro degradation reactions, and decay was monitored over a time course. As in previous experiments, the decay of full-length gA6[14]-17U was more rapid than decay of gA6[14] (Fig. 4). In contrast to the full-length oligouridylated gA6[14], which decayed with an average half life of 5.0 ± 1.5 min, gA6[14]-22A degraded at a relatively slow rate comparable to decay of gA6[14]NT (Fig. 4). In the experiment presented in Fig. 4, gA6[14]-22A decayed with an average half life of 14.7 ± 4.1 min compared to 14.8 ± 4.3 min for gA6[14]NT. Furthermore, decay of gA6[14]-17U and gA6[14]-22A produced different size intermediate products (I versus I^*, Fig. 4). Whereas decay of oligouridylated gA6[14] produced a band 2–4 nucleotides shorter than gA6[14]NT, the decay product of polyadenylated gA6[14] was slightly larger, ending at or 1 nucleotide past the end of the poly(A) tail. Although the gA6[14]-22A decay intermediate is present in the starting material, quantification indicates that it accumulates over time. Overall, these results indicate that rapid decay of the 3′ end of gRNAs requires an oligo(U) tail.

The oligo(U) tails on gRNAs reportedly vary from 5 to 24U residues [7]. To determine the minimal tail length capable of supporting the initial rapid step of gRNA decay, we generated two additional RNAs. RNAs derived from gA6[14] were synthesized with a 5- or 10-nucleotide oligo(U) extension. Degradation of these RNAs was compared to that of gA6[14]NT and gA6[14]-17U in vitro. As with our previous experiments, full-length gA6[14]-17U degraded more rapidly than gA6[14]NT (Fig. 5). RNAs with 3′ tails composed of 5 or 10 uridines degraded more rapidly than gA6[14]NT but more slowly than gA6[14]-17U (Fig. 5). In the experiments presented in Fig. 5, half lives were 11.9 ± 4.7, 17.7 ± 7.2, 22.8 ± 4.0, 32.1 ± 4.3 min for gA6[14]-17U, gA6[14]-5U, gA6[14]-10U and gA6[14]NT, respectively. Statistical analysis indicates that the half lives of gA6[14]-5U and gA6[14]-10U are statistically different from that of gA6[14]NT ($p < 0.05$). The half lives of gA6[14]-10U and gA6[14]-17U are also statistically different from each other ($p < 0.05$). The half life of gA6[14]-5U is not statistically different from either gA6[14]-10U or gA6[14]-17U ($p > 0.10$). Interestingly, decay of RNAs with 5- or 10-uridine extensions produced an intermediate product the size of gA6[14]NT, in contrast to the smaller intermediate previously described for gA6[14]-17U (I^* versus I, Fig. 5). From these experiments we conclude that rapid degradation of the 3′ end of gRNA can be achieved with an oligo(U) tail as short as 5 nucleotides, but the

rate of decay is faster and proceeds further into encoded portion of the gRNAs with a 17 nucleotide oligo(U) tail.

3.4. RNA competition experiments implicate an enzyme with high affinity for oligouridylated gRNA

To begin to examine the biochemical properties of the enzyme(s) that mediates rapid decay of the gRNA 3′ end and slow decay of the gRNA body, we examined the effect of excess, unlabeled RNA on each of these decay events. Unlabeled poly(U), poly(C), or tRNA was added to degradation reactions in 1, 5, or 10 fold molar excess compared to gA6[14]-17U or gA6[14]NT (Fig. 6). Surprisingly, addition of poly(U) to degradation reactions had a greater inhibitory effect on decay of gA6[14] lacking a oligo(U) tail (gA6[14]NT) than it did on decay of gA6[14]-17U (Fig. 6). Decay of gA6[14]NT was completely inhibited at all concentrations of poly(U) tested. In contrast, decay of gA6[14]-17U was inhibited only 26% by equimolar poly(U) and 74% at 5-fold molar excess poly(U). Even at 10-fold molar excess poly(U), decay of gA6[14]-17U was not completely inhibited, but was reduced by 86% compared to reactions performed in the absence of competitor. Decay of gA6[14]-17U and its non-tailed counterpart, gA6[14]NT, differed even more dramatically when inhibition by other competitor RNAs were tested. Decay of gA6[14]-17U was essentially refractory to the addition of up to10-fold molar excess poly(C), whereas decay gA6[14]-NT was completely inhibited by a 5-fold excess of the same ribohomopolymer. Similarly, addition of tRNA had only a very slight effect on the decay of gA6[14]-17U, causing only 15–20% inhibition even at 10-fold molar excess. Decay of gA6[14]-NT was more sensitive to tRNA addition, exhibiting ∼50% inhibition even at 5-fold molar excess of tRNA. These competition results strongly suggest that decay of the oligo(U) tail and decay of the gRNA body are mediated by distinct ribonucleases with differing specificities. The enzyme that degrades the body of the gRNA is relatively non-specific. It is significantly inhibited by all three competitors, displaying a slight preference for poly(U) > poly(C) > tRNA. In contrast, the enzyme responsible for rapid decay of the oligo(U) tail is only significantly inhibited poly(U). Moreover, much higher concentrations of poly(U) are necessary to achieve similar levels of competition of gA6[14]-17U compared to gA6[14]-NT. Despite the moderate sensitivity of gA6[14]-17U to poly(U) competition, is unlikely that decay of the gRNA oligo(U) tail is mediated by a U-specific exoribonuclease for two reasons. First, the initial rapid decay step proceeds a few nucleotides into the encoded (non-U) gRNA sequence. Second, and most importantly, mRNAs bearing 3′ oligo(U) tails are almost completely stable in the same system [22]. Together, our results suggest that gRNA decay is initiated by an enzyme that preferentially recognizes oligouridylated gRNAs.

4. Discussion

In this study, we utilized in organello pulse chase and in vitro turnover assays to examine the mechanisms of gRNA turnover. Our data indicate the presence of a gRNA-specific decay pathway that takes place using a biphasic mechanism. The first step in gRNA decay involves the rapid removal of the 3′ oligo(U) tail and a few encoded nucleotides. This step is followed by a slower decay of the gRNA body. Elements of both the oligo(U) tail and the encoded potion of the gRNA are apparently required to trigger the initiation of this pathway.

When monitoring [α^{32}P]-UTP labeling of RNA in isolated mitochondria, we observed disappearance of the majority of the gRNA label within 1 min. However, the remaining approximately 30% of the gRNA population remains stable up to 30 min. Using similar methods to study oligouridylation of gRNAs, McManus et al. observed stable oligouridylated gRNAs in two glycerol gradient fractions of [α^{32}P]-UTP labeled mitochondria [18]. These fractions, complex I and complex II, are 19S and 35S to 40S ribonucleoprotein complexes which contain proteins involved in RNA editing. Complex II has been proposed to be the fully assembled active editing complex [18]. In these two complexes, all of the labeled gRNAs remained stable up to 30 min [18]. In fact, gRNAs present in complex II actually grew longer, indicating the presence of an active TUTase. In our studies, we observe an increase in the average size of the stable gRNA population over time, suggesting that protected gRNAs may already contain oligo(U) tails (Fig. 1). Although we do not observe lengthening of gRNAs in isolated mitochondria, the stable population we do observe has a similar size distribution on denaturing acrylamide gels as those previously observed in complexes I and II on glycerol gradients. These results suggest that the protected gRNA populations in our system may be bound to either or both editing-related complexes. Based on our in vitro studies, we conclude that the rapid disappearance of the remaining gRNA that we observe in isolated mitochondria represents primarily rapid decay of labeled gRNA oligo(U) tails. Together, these observations suggest that rapid degradation, or at least deuridylation, of gRNAs takes place either prior to their use in editing and/or after their dissociation from the editing complex.

Experiments utilizing our in vitro degradation system indicate a two-step mechanism of gRNA decay entailing rapid removal of the oligo(U) tail and few encoded nucleotides followed by slower degradation of the body of the gRNA. The slower decay of the encoded portion of the gRNA occurs independently of the presence of an oligo(U) tail. This pattern of gRNA decay was shown to be very similar for two different gRNAs. To examine the role of tail length and sequence in the first, rapid step of gRNA decay, we monitored degradation of several different gRNAs in comparison to decay of gA6[14]-17U and non-tailed gA6[14]. gRNAs bearing a 3′ oligo(A) tail were relatively stable compared to all 3′ uridylated gRNAs. Decreasing the oligo(U) tail length to also lead to a somewhat slowed rate of 3′ decay compared to gRNAs with a 17U tail. Ultimately, on gRNAs with either 5 uridine or 22 adenosine tails, the 3′ tail was removed. However, the stable intermediate that formed with either 22A tailed or 5U tailed gRNAs had fewer encoded nucleotides removed compared to degradation intermediates of gA6[14]-17U. Therefore, shortening the oligo(U) tail or altering the tail sequence to an oligo(A) stretch both prevents decay of encoded nucleotides and slows decay of the 3′ extension to

different extents. These differences may result from loading of a different nuclease complex, potentially lacking a processivity factor and/or a helicase, onto 5 uridine or oligo(A) tailed gRNAs compared to those with a 17 uridine stretch. Alternatively, the same nuclease may act on each of these molecules, but may not load as stably on gRNAs with different length or sequence tails. Less stable binding could result in an overall decrease in degradation rate. Furthermore, if a nuclease is less stably bound, its premature release from the gRNA may lead to decay intermediates that are longer than those produced by a more processive nuclease.

Several lines of evidence suggest the existence of a gRNA-specific degradation pathway. First, in isolated mitochondria, oligo(U) tails on rRNAs are stable over time, whereas gRNA oligo(U) tails are very rapidly removed. In vitro, gRNA oligo(U) tails are also rapidly degraded. In stark contrast, we previously showed that oligo(U) tails on unedited, mitochondrially encoded mRNAs are very slowly degraded in the same in vitro system [22]. Thus, rapid removal of the gRNA oligo(U) tail is not due to the action of a promiscuous U-specific exoribonuclease present in our mitochondrial extracts. Rather, these results suggest that decay of the gRNA oligo(U) tail is dependent on the presence of encoded gRNA sequences. We also demonstrated here that gRNAs bearing a 3′ oligo(A) tail decay at the same rate as non-tailed gRNAs. In contrast, decay of both unedited mRNAs and reporter RNAs is markedly stimulated by polyadenylation in the same system [22]. These results indicate that distinct regulatory factors and/or ribonucleases assemble on different mitochondrial RNA populations. Taken together, these results suggest that the nuclease or nuclease-containing complex that rapidly degrades gRNA oligo(U) tails recognizes a combination of the oligo(U) tail and a common gRNA feature apart from 3′ oligouridylation. The latter feature is likely to entail elements of secondary and/or tertiary structure [9,28]. Future experiments will address the gRNA-specific signals that trigger the rapid first step of gRNA decay.

The data reported here are the first to address the biochemical mechanism of gRNA turnover. It is important to note that the gRNA decay pathway identified here may be only one of several mechanisms by which gRNAs undergo turnover in *T. brucei* mitochondria. gRNAs may be degraded at several points, including during differentiation or following their use in the editing reaction. Different ribonucleases and/or accessory factors may well be involved in gRNA decay under different circumstances. Our establishment of an in vitro system for studying gRNA decay provides a starting point for future detailed analyses of *cis*- and *trans*-acting factors involved in gRNA turnover. This information can ultimately be used to determine whether multiple gRNA decay pathways exist and, if so, whether they share common components. The system described here will also allow us to differentiate between the turnover pathways for gRNA and mRNA, and to determine whether decay of these RNA species occurs through the action of different ribonucleases or whether it involves a common nuclease with different *trans*-acting factors. Understanding the factors involved gRNA turnover will lead to insight into the role of this process in trypanosome mitochondrial gene regulation.

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