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Opposing Effects of Polyadenylation on the Stability of Edited and Unedited Mitochondrial RNAs in *Trypanosoma brucei*

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Mitochondrial RNAs in *Trypanosoma brucei* undergo posttranscriptional RNA editing and polyadenylation. We previously showed that polyadenylation stimulates turnover of unedited RNAs. Here, we investigated the role of polyadenylation in decay of edited RPS12 RNA. In in vitro turnover assays, nonadenylated fully edited RNA degrades significantly faster than its unedited counterpart. Rapid turnover of nonadenylated RNA is facilitated by editing at just six editing sites. Surprisingly, in direct contrast to unedited RNA, turnover of fully edited RNA is dramatically slowed by addition of a poly(A)$_{20}$ tail. The same minimal edited sequence that stimulates decay of nonadenylated RNA is sufficient to switch the poly(A) tail from a destabilizing to a stabilizing element. Both nucleotide composition and length of the 3′ extension are important for stabilization of edited RNA. Titration of poly(A) into RNA degradation reactions has no effect on turnover of polyadenylated edited RNA. These results suggest the presence of a protective protein(s) that simultaneously recognizes the poly(A) tail and small edited element and which blocks the action of a 3′-5′ exonuclease. This study provides the first evidence for opposing effects of polyadenylation on RNA stability within a single organelle and suggests a novel and unique regulation of RNA turnover in this system.

Trypanosoma brucei is a unicellular, flagellated, parasitic protozoan that causes African sleeping sickness in humans and nagana in domestic livestock. It contains a single large mitochondrion, whose gene expression and metabolic activity are regulated throughout its life cycle. Procyclic (insect) form parasites contain a fully functional mitochondrion that carries out oxidative phosphorylation (3, 29, 42). In contrast, bloodstream (mammalian) form trypanosomes exhibit a high rate of glycolysis and contain an underdeveloped mitochondrion (3, 29, 42). Similar to other eukaryotes, the genes for the mitochondrial proteins are encoded both in the nuclear and mitochondrial genomes.

Since transcription of *T. brucei* mitochondrial DNA generates polycistronic transcripts (14, 19, 22, 33), posttranscriptional mechanisms of gene expression and their regulation are of prime importance. Maturation of mitochondrial RNAs requires several posttranscriptional processing steps, including precursor cleavage, RNA editing, and polyadenylation. Polycistronic precursors must be endonucleolytically cleaved and possibly exonucleolytically trimmed to create the correct 5′ and 3′ ends of individual monocistronic RNAs. In addition, 12 of the 18 transcripts in *T. brucei* mitochondria require editing, which involves specific insertion and deletion of uridine residues, to create translatable RNAs (for recent reviews see references 27, 37, 38, and 40). mRNAs are also extended at their 3′ ends by polyadenylation. The length of poly(A) tail has been found to correlate with the edited status of the RNA. While unedited RNAs contain either no or short (~20 nucleotides [nt]) poly(A) tails, edited RNAs, including partially and fully edited RNAs, contain either short or long (~120 to 200 nt) poly(A) tails (2, 11, 30, 33, 34, 39). In addition, the poly(A) tail lengths on specific mitochondrial mRNAs are regulated throughout the life cycle of trypanosomes (2, 34), suggesting that polyadenylation of mitochondrial RNAs may regulate their gene expression during trypanosome development.

The poly(A) tail plays a critical role in modulating RNA stability in many systems. In eukaryotic cytoplasm, the poly(A) tail in conjunction with poly(A)-binding protein I increases the stability of many mRNAs (1, 15, 28, 43). On the contrary, in bacteria and chloroplasts, poly(A) tails act as mRNA-destabilizing elements (4, 8, 24, 25, 36). In mitochondria, the effects of polyadenylation on RNA stability differ dramatically from organism to organism (18). In human mitochondria, there is an apparent correlation between shortened poly(A) tails and decreased RNA stability, suggesting that polyadenylation may serve as a stabilizing element in this system (41). Conversely, in plant mitochondria, both in vivo and in vitro data have shown that polyadenylation accelerates decay of mRNAs (16, 17, 23). In yeast, mitochondrial RNAs are not polyadenylated and, rather, an encoded dodecamer sequence is involved in controlling RNA stability in this system (7). Our laboratory previously used in organello pulse-chase experiments to identify an RNA decay pathway in *T. brucei* mitochondria in which polyadenylation stimulates RNA turnover (31). We subsequently developed an in vitro RNA turnover system by which we were able to recapitulate poly(A)-specific ribonucleolytic activity toward unedited transcripts and further characterize these pathways in vitro (35). However, in our previous in organello and in vitro experiments, only the decay of unedited RNAs was studied. To date, it is still largely unknown whether or how the stability of partially and fully edited RNAs is regulated in *T. brucei* mitochondria. Although RNA editing and polyadenylation are coordinated in *T. brucei* mitochondria, the relationship between differential polyadenylation and edited RNA stability has not been previously addressed.

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TABLE 1. Primers used for PCR to generate templates for in vitro transcription

RNA	Polarity[a]	Primer sequence[b]
All RPS12	S	5′-TGTAATACGACTCACTATAGGGCTAATACACTTTTGATAACAAACTAAAGTAAA-3′
RPS12UE	AS	5′-AAAAACATATCTTATTCT-3′
RPS12FE	AS	5′-AAAAACATATCTTATATCTAAATCTAACTTACAATACGT-3′
RPS12PE6	AS	5′-AAAAACATATCTTAT-3′
RPS12PE45	AS	5′-AAAAACATATCTTAT-3′
All RPS12-20A	AS	5′-TTTTTTTTTTTTTTTTTTTTAAAAACATATCTTAT-3′
RPS12FE-5A	AS	5′-TTTTTAAAAACATATCTTAT-3′
RPS12FE-20U	AS	5′-AAAAAAAAAAAAAAAAAAAAAAAACATATCTTAT-3′
RPS12FE-20AU	AS	5′-TTTTTTTAAAATTTTTTTTTAAAAACATATCTTTAT-3′

[a] S, sense; AS, antisense.
[b] Underlined sequence indicates the T7 promoter region.

In this study, we examined the turnover of edited mitochondrial RNAs using our established in vitro system (35). We found that in the absence of a poly(A) tail, edited RNAs degrade much more rapidly than their unedited counterparts. This rapid turnover also occurs on partially edited RNAs in which only ~10% of editing is completed, thereby defining a small *cis*-acting edited element that facilitates RNA decay. Most surprisingly, we found that while a poly(A)$_{20}$ tail stimulates decay of unedited mitochondrial transcripts, the same 3′ modification impedes degradation of edited transcripts. The small edited region on the aforementioned partially edited RNA is sufficient to switch a poly(A)$_{20}$ tail from a destabilizing to a stabilizing element. Neither a poly(A)$_5$ tail nor a poly(U)$_{20}$ tail stabilizes edited transcripts, indicating that both length and nucleotide composition are important for the polyadenylation-mediated stabilization of edited RNAs. Titration of poly(A) homopolymer into RNA turnover reactions does not stimulate decay of edited polyadenylated transcripts, suggesting that stabilization of edited RNAs by poly(A)$_{20}$ tails in *T. brucei* mitochondria is mediated by a mechanism different from that in eukaryotic cytoplasm. Finally, degradation of edited mitochondrial transcripts involves both endo- and exoribonucleases, and the action of the latter is impeded by the presence of a poly(A)$_{20}$ tail. The opposing effects of polyadenylation on the stability of unedited and edited RNAs in *T. brucei* mitochondria suggest a novel and unique regulation of RNA turnover in this system.

MATERIALS AND METHODS

Cell culture and mitochondrial extract preparation. Procyclic form *T. brucei brucei* clone IsTaR1 stock EATRO 164 was grown as described previously (6). Mitochondrial vesicles were isolated on 20-to-35% linear Percoll gradients and stored at −80°C according to the method of Harris et al. (20). Partially purified mitochondrial extract used in the in vitro degradation assays was prepared as previously described (35). Briefly, Percoll gradient-purified mitochondrial vesicles were pelleted and lysed in nuclease buffer (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) containing 0.2% Nonidet P-40 at 4°C for 10 min at a concentration of 2×10^{10} cell eq/ml. The lysate was centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was briefly washed with nuclease buffer containing 0.2% Nonidet P-40 and again centrifuged at 100,000 × *g* for 30 min at 4°C. The pellet was then resuspended in nuclease buffer containing 1 M KCl and 0.2% Nonidet P-40 at a concentration of 2×10^{10} cell eq/ml to extract peripheral membrane proteins. This resuspension was then homogenized with a B-type pestle and Dounce homogenizer and then incubated for 1 h at 4°C with constant rotation. The homogenized extract was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was dialyzed against nuclease buffer containing 50 mM KCl overnight at 4°C and stored at −80°C for later use.

RNA substrate preparation. All RNAs used in the in vitro degradation assays were synthesized by in vitro transcription from PCR-generated templates, created using the primers listed in Table 1. The sense primer (RPS12-5′-T7) contains a T7 promoter region and a sequence complementary to the 5′ never-edited region of all RPS12 RNA derivatives (unedited, partially edited, and fully edited RNAs). The antisense primers contain a region complementary to the RNA of interest and an extension of appropriate length and composition to produce RNAs indicated in Table 1. The plasmid pRPS12-U (31) was used as a PCR template to generate a 221-nt RPS12UE (where UE is unedited) RNA without or with a 3′ 20-A tail. The plasmid pRPS12-E (31) served as a PCR template to generate a 325-nt RPS12FE (where FE is fully edited) without or with 3′ extensions. Clones 3p-23 and 1p-25 from a previous study in our laboratory (30) contain partially edited RPS12 DNA sequences and served as PCR templates for RPS12PE45 and RPS12PE6, respectively, without or with 3′ 20-A tails.

RNAs were synthesized from the PCR products described above with T7 RNA polymerase in the presence of [α-^{32}P]GTP using the Megascript in vitro transcription kit (Ambion, Inc.). Alternatively, RNAs were end labeled as previously described (35). Labeled RNAs were separated on 6% acrylamide–7 M urea denaturing gels and visualized by UV shadowing or autoradiography. Full-length RNAs were excised, eluted from the gel, and recovered by isopropanol precipitation.

RPS12FE-200A RNAs were synthesized by incubating internally labeled RPS12-20A RNAs with yeast poly(A) polymerase (U.S. Biochemicals, Inc.) and ATP. The following reaction conditions were optimized for the synthesis of RPS12FE RNA with poly(A) tail length ranging from 150 to 250 nt. A standard 50-μl reaction mixture contained 30 pmol of labeled RNA, 140 μM ATP, 1 μl of RNaseOUT (40 U/μl; Invitrogen, Inc.), and 1× poly(A) polymerase reaction buffer (20 mM Tris-HCl [pH 7.0], 50 mM KCl, 700 μM $MnCl_2$, 200 μM EDTA, 100 μg of acetylated bovine serum albumin/ml, and 10% glycerol). Reaction mixtures were incubated at 30°C for 30 min and then stopped by the addition of 2 μl of 0.5 M EDTA. Labeled RNAs were again separated on 6% acrylamide–7 M urea denaturing gels and visualized by autoradiography. RNAs with tail lengths between 150 and 250 nt, as judged by RNA Century Marker Plus (Ambion, Inc.), were excised, eluted from the gel, and recovered by isopropanol precipitation.

In vitro RNA degradation assay. In vitro RNA degradation assays were set up as previously described (35) with slight modifications. Briefly, a 25-μl reaction volume contained nuclease buffer, 10 mM $MgCl_2$, 1 mM UTP, 1 to 2.5 μg of partially purified mitochondrial extract, and 2.5 pmol of RNA. To ensure proper folding, all RNAs were heated for 1 min at 95°C and then slowly cooled down to room temperature for at least 10 min prior to use. Reaction mixtures were incubated at 27°C for the times indicated and stopped by the addition of 5 μl of stop buffer (50 mM EDTA and 0.2% sodium dodecyl sulfate). RNAs were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and 20 μl of the aqueous layer was added to 20 μl of 90% formamide loading buffer. Samples were heated for 5 min at 95°C. Equal volumes of each reaction mixture were then analyzed by electrophoresis on 6% acrylamide–7 M urea gels followed by autoradiography or phosphorimager analysis. Nonsaturated autoradiographs were analyzed using a Bio-Rad GS-700 imaging densitometer and Multi-Analyst software (version 1.1). Percent full-length RNA remaining was determined by analyzing the density of areas corresponding to full-length RNAs. Phosphorimager analysis was performed on a Bio-Rad Personal FX PhosphorImager using Quantity One software (version 4.2.1).

TLC. Products of in vitro degradation reactions were analyzed by thin-layer chromatography (TLC) as previously described (35).

RESULTS

Effects of edited *cis*-acting elements on mitochondrial RNA turnover. We previously studied the turnover of mitochondrial transcripts using in organello pulse-chase experiments and

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RPS12UE      1 CUAAUACACUUUUGAUAACAAACUAAAGUAAA A A    G        GCG A G GAUU  44
RPS12PE6     1 CUAAUACACUUUUGAUAACAAACUAAAGUAAA A A    G        GCG A G GAUU  44
RPS12PE45    1 CUAAUACACUUUUGAUAACAAACUAAAGUAAA A A    G        GCG A G GAUU  44
RPS12FE      1 CUAAUACACUUUUGAUAACAAACUAAAGUAAAuAuAuuuuGuuuuuuuuGCGuAuGuGA*U  60

RPS12UE     45 UUUUG A GUG  G  G   ACUG    G    A   G    A G  A  A A GAG CCGUUC  76
RPS12PE6    45 UUUUG A GUG  G  G   ACUG    G    A   G    A G  A  A A GAG CCGUUC  76
RPS12PE45   45 UUUUG A GUG  G  G   ACUG    G    A   G    A G  A  A A GAG CCGUUC  76
RPS12FE     61 UUUUGuAuG*GuuGuuGuuuAC*GuuuuGuuuuAuuuGuuuuAuGuuAuuAuAuGAGuCCG**C  120

RPS12UE     77 GA  GCCCAG  CCG    G A A CCGACG G A  G A    G CUUCUUUUG A    A  UA A AA  117
RPS12PE6    77 GA  GCCCAG  CCG    G A A CCGACG G A  G A    G CUUCUUUUG A    A  UA A AA  117
RPS12PE45   77 GA  GCCCAG  CCGuuuuG AuAuCCGACG G A  GuAuuuuGuCU*C****GuAuuuuAuuUAuAuAA  133
RPS12FE    121 GAuuGCCCAGuuCCG    GuA A CCGACGuGuAuuGuAu   G C**C****GuAuuuuAuuUAuAuAA  175

RPS12UE    118     G   GGA G  GCG  G        G  G    A  GG   AG  A GUUUCA  A   A  A  143
RPS12PE6   118     G   GGA G  GCG  G        G  G    A  GG   AG  A GUUUCA  A   A  A  143
RPS12PE45  134 uuuuGuuuGGAuGuuGCGuuGuuuuuuuuGuuGuuuuAuuGGuuuAGuuAuG**UCAuuAuuuAuuA  197
RPS12FE    176 uuuuGuuuGGAuGuuGCGuuGuuuuuuuuGuuGuuuuAuuGGuuuAGuuAuG**UCAuuAuuuAuuA  240

RPS12UE    144  AGAUUUGGGUGG GG    G  GA   ACCCUUUGUUUUG GUUAAAG A  A   ACAUCGUUUA  G AAG   196
RPS12PE6   144  AGAUUUGGGUGG GG    G  GA   ACCCUUUGUUUUG GUUAAAG A  AuuuACA*CG*UUA  G AAGuu 200
RPS12PE45  198 uAGA***GGGUGGuGGuuuuGuuGAuuuACCC***G****GuG*UAAAGuAuuAu  ACA*CG**UAuuGuAAGuu 257
RPS12FE    241 uAGA***GGGUGGuGGuuuuGuuGAuuuACCC***G****GuG*UAAAGuAuuAu  ACA*CG**UAuuGuAAGuu 300

RPS12UE    197 AGAUUUUAGA AUAAGAUAUGUUUUU  221
RPS12PE6   201 AGA*UUUAGAuAUAAGAUAUGUUUUU  225
RPS12PE45  258 AGA*UUUAGAuAUAAGAUAUGUUUUU  282
RPS12FE    301 AGA*UUUAGAuAUAAGAUAUGUUUUU  325
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FIG. 1. RNA sequences of RPS12 transcripts of various editing statuses used in this study. Editing of RPS12 RNA involves 132 uridine insertions and 28 uridine deletions at 77 editing sites. Lower case u's represent uridine residues inserted during editing, and asterisks represent uridine residues deleted during RNA editing. Letters highlighted in gray represent junction regions containing edited sequence that does not match fully edited RNA. RPS12UE, unedited; RPS12PE6, partially edited at 6 editing sites; RPS12PE45, partially edited at 45 editing sites; RPS12FE, fully edited.

identified an RNA decay pathway in which polyadenylation stimulated RNA turnover (31). However, due to the limited sensitivity of this assay, only the degradation of unedited mitochondrial transcripts was examined. We subsequently developed an in vitro RNA turnover system and further characterized the poly(A)-stimulated turnover of unedited RNAs (35). The goal of the present study was to identify the factors that mediate turnover of edited mitochondrial RNAs. Again, we employed our in vitro system (35). As we previously reported, the absolute level of nuclease activity varied widely between preparations, and the activity was quite labile (35). Therefore, although the relative degradation rates of various RNAs were highly reproducible, the time course of RNA degradation differed between experiments. For this reason, we present representative experiments in the figures below. To establish the reproducibility of our findings, the differences in half-life between RNAs are reported as the mean ± standard deviation of at least three experiments. We chose the pan-edited ribosomal protein S12 (RPS12) RNA as the substrate in this study. The primary sequences of the four differentially edited versions of RPS12 RNA used in this study are shown in Fig. 1 (30, 33).

To determine whether edited RNA sequences act as *cis*-acting regulatory elements in RNA decay apart from 3′ poly(A) tracts, we first compared the degradation rates of nonadenylated fully edited RPS12 RNAs (RPS12FE) and unedited RPS12 RNAs (RPS12UE) (Fig. 2). In this assay, in vitro-transcribed, body-labeled RNAs were incubated with partially purified mitochondrial lysates (35), and RNA degradation was monitored by gel electrophoresis followed by autoradiography. Time course experiments showed that fully edited RPS12 RNAs degraded significantly faster than their unedited counterparts (Fig. 2). In the experiment presented, RPS12FE degraded with a half-life of ~20 min, whereas RPS12UE degraded more slowly with a half-life longer than 120 min (Fig. 2). Over several experiments, we observed a 4.1-fold ± 0.6-fold ($n = 5$) difference in half-life between RPS12FE and RPS12UE. As shown in Fig. 2, the length of RPS12FE RNAs decreased slightly over time but accumulation of stable degradation intermediates was rarely observed and required long exposure.

Next, we set out to determine the degree of editing required to facilitate rapid decay of RPS12 transcripts. This is of particular physiological significance since the steady-state RNA population contains a large percentage of RNAs that are edited to various extents at their 3′ ends and unedited at their 5′ ends (11, 21, 30, 39). We previously obtained a battery of RPS12 clones that were edited to various extents through oligo(dT) column selection and reverse transcription-PCR (RT-PCR) (30). For our purposes, two partially edited RPS12 RNAs were chosen, and their schematic diagram is shown in Fig. 3A. RPS12PE45 was edited at 45 of 77 editing sites (~60% of the 3′ end), which results in addition of 84 uridines and deletion of 22 uridines (Fig. 1). Time course experiments showed that RPS12PE45 degraded more rapidly than RPS12UE, with a half-life similar to that of RPS12FE (Fig. 3B). Over several experiments, we observed a 2.6-fold ± 0.3-fold ($n = 3$) difference in half-life between RPS12UE and RPS12PE45. These data indicate that complete editing is not required to facilitate rapid decay of nonadenylated RPS12

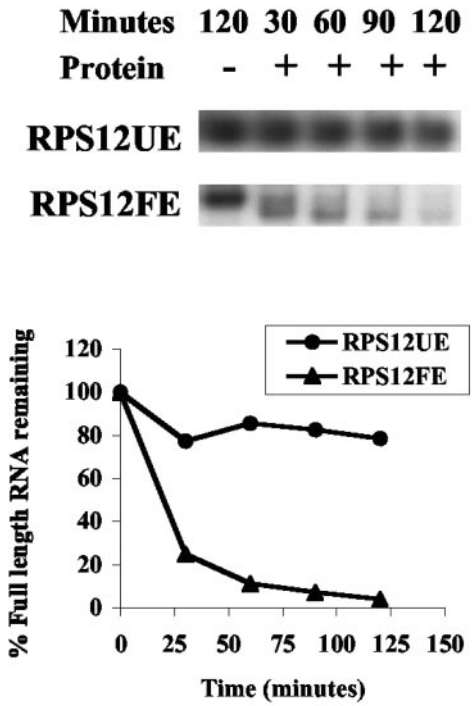

FIG. 2. Fully edited RPS12 transcripts degrade more rapidly than their unedited counterparts. RPS12UE and RPS12FE RNAs were in vitro transcribed and internally labeled with [α-^{32}P]GTP. These RNAs were incubated with partially purified mitochondrial fractions at 27°C for the indicated times. (Upper panel) Products were resolved on a 7 M urea–6% acrylamide gel and visualized by autoradiography. (Lower panel) Percent full-length RNA remaining was determined by densitometry of a nonsaturated autoradiograph and was plotted for each time point.

RNA. To further narrow down the degree of editing sufficient to stimulate decay, the turnover of RPS12PE6 was examined (Fig. 3B). RPS12PE6 was edited at 6 of 77 editing sites (~10% of the 3′ end), which involved addition of six uridines and deletion of three uridines (Fig. 1). Surprisingly, this small edited element, which only slightly modified the 3′ end of RPS12 RNA, strongly destabilized this transcript to a similar extent as RPS12FE and RPS12PE45. In the experiments presented in Fig. 3B, RPS12PE6 degraded with a half-life of ~20 min, while the half-life of RPS12UE was greater than 120 min. Over several experiments, we determined that the fold difference between half-lives of RPS12UE and RPS12PE6 was 3.3 ± 0.8 (n = 6). These results demonstrate that edited *cis*-acting sequences encompassing only six editing sites are sufficient to serve as destabilizing elements for RPS12 transcripts in this system.

Effect of polyadenylation on turnover of edited RNAs. In vivo, unedited RPS12 transcripts are either nonadenylated or contain short (~20-nt) poly(A) tails, whereas edited RPS12 RNAs contain both short (~20-nt) and long (120- to 200-nt) poly(A) tracts (30, 33). We previously reported that poly(A)$_{20}$ tails serve as destabilizing elements at the 3′ end of unedited RPS12 RNAs (35). Here, we determined the effect of poly(A)$_{20}$ tails on the stability of edited RPS12 RNAs (Fig. 4). Degradation of fully edited RPS12 RNAs with or without a poly(A)$_{20}$ tail (RPS12FE-20A and RPS12FE, respectively) was measured over a time course. Turnover rates of their unedited counterparts, measured in parallel, are shown for comparison (RPS12UE-20A and RPS12UE, respectively). As expected, addition of a poly(A)$_{20}$ tail to RPS12UE RNAs facilitated RNA degradation (Fig. 4A). In striking contrast, addition of a 20-A tail to

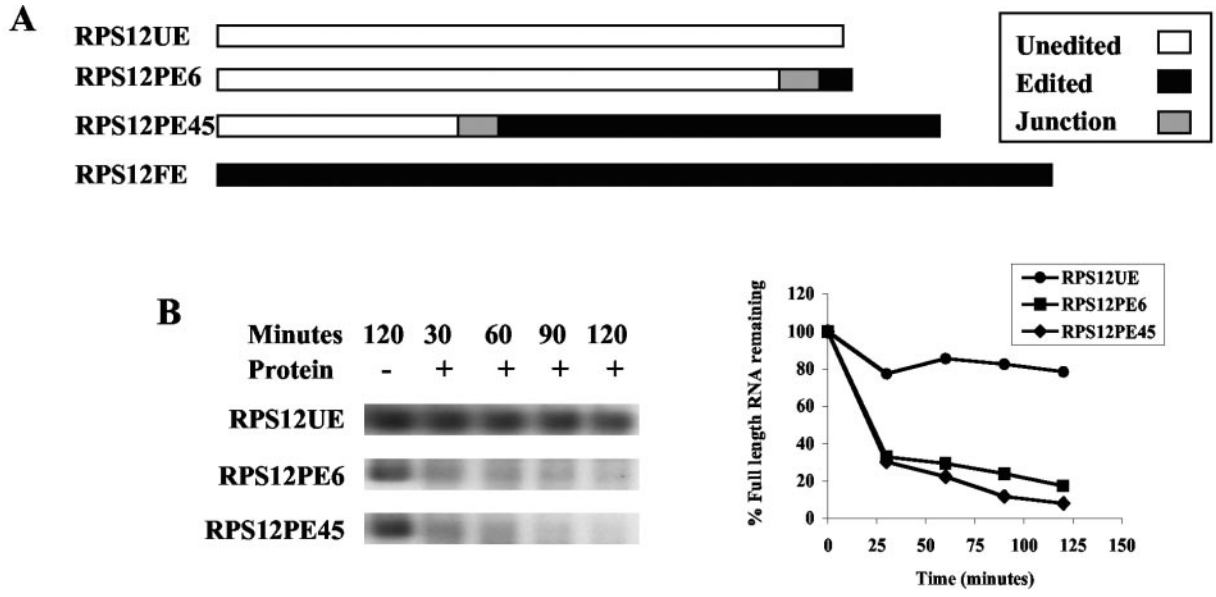

FIG. 3. An edited *cis*-acting sequence encompassing only six editing sites is sufficient to facilitate decay of RPS12 transcripts. (A) Schematic representation of unedited, fully edited, and two partially edited RPS12 RNAs (shown 5′ to 3′, not to scale). RPS12PE6 is edited at 6 of 77 editing sites (~10% edited), and RPS12PE45 is edited at 45 of 77 editing sites (~60% edited). Black regions indicate fully edited sequence. Gray indicates junction regions containing partially edited sequence. (B) Internally labeled RPS12UE, RPS12PE6, and RPS12PE45 transcripts were incubated with partially purified mitochondrial fractions at 27°C for the indicated times. Products were resolved on a 7 M urea–6% acrylamide gel and visualized by autoradiography (left panel). Percent full-length RNA remaining was determined by densitometry of a nonsaturated autoradiograph and was plotted for each time point (right panel).

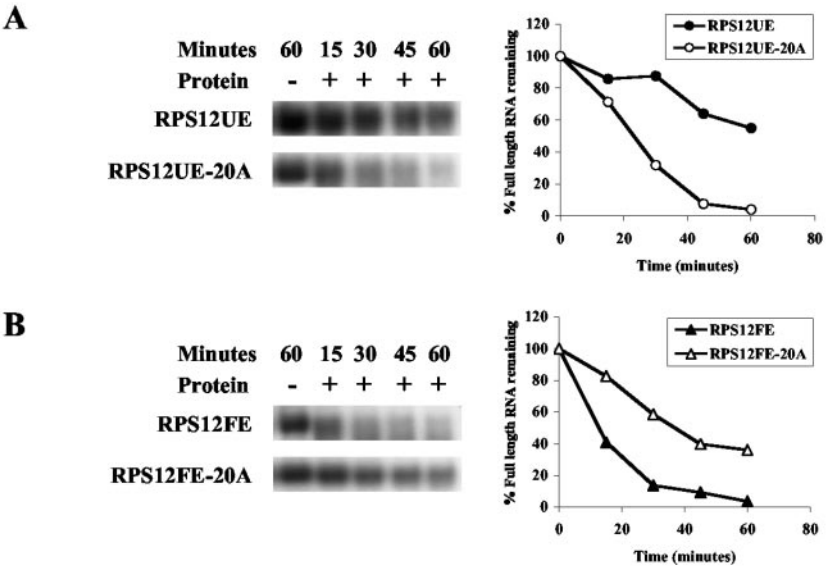

FIG. 4. A poly(A)$_{20}$ tail destabilizes unedited RPS12 RNAs but stabilizes fully edited RPS12 RNAs. RPS12UE (A) and RPS12FE (B) RNAs with or without a 3′ 20-A tail were synthesized in vitro. RNA degradation reactions were performed as described in the legend for Fig. 2. The left panels show the autoradiographs. The percent full-length RNA remaining for each time point as determined by densitometry is plotted in the right panels.

RPS12FE strongly impeded decay of this RNA. In the experiment presented in Fig. 4B, RPS12FE-20A degraded with a half-life of ~40 min, whereas RPS12FE degraded with a half-life of ~15 min. Over several experiments, we observed a 3.3-fold ± 0.7-fold (n = 7) difference in half-life between RPS12FE and RPS12FE-20A. This experiment demonstrates that a 3′ poly(A) tail has opposite effects on the stability of fully edited and unedited RPS12 RNAs.

Next, we determined the extent of editing that is necessary to switch a poly(A)$_{20}$ tail from a destabilizing to a stabilizing element. Again, we utilized RPS12PE45 and RPS12PE6 RNAs, which are ~60 and 10% edited at their 3′ ends. As shown in Fig. 5B and C, both RPS12PE45-20A and RPS12PE6-20A had two- to threefold longer half-lives than their nontailed counterparts, while RPS12UE-20A was degraded with a two- to threefold shorter half-life than nontailed RPS12UE, as expected (Fig. 5A). Data from several experiments showed that RPS12PE45-20A had a 2.9-fold ± 0.5-fold (n = 3) longer half-life than RPS12PE45, and RPS12PE6-20A had a 3.1-fold ± 0.4-fold (n = 3) longer half-life than RPS12PE6. We previously reported that addition of a poly(A)$_{20}$ tail markedly destabilizes a variety of RNAs in this system, including mitochondrial-encoded, nuclear-encoded, or plasmid-encoded RNAs (35). Thus, the poly(A) tail alone is sufficient to act as a destabilizing element in our in vitro system. The demonstration that the poly(A)$_{20}$ tail stabilizes edited transcripts suggests that these 20-A-tailed edited RNAs may be protected by a protein or protein complex that recognizes poly(A) tails in conjunction with edited *cis*-acting sequences.

Effects of two additional in vivo poly(A) tails on turnover of edited RNAs. Edited RNAs are generally present in two populations, one with ~20-nt tails and another with ~200-nt tails (2, 34). Accordingly, edited RPS12 RNAs with ~200-nt tails have been reported (30, 33). To determine the effect of this long poly(A) tail on stability of edited RNAs, RPS12FE RNAs containing poly(A) tails ranging from 150 to 250 nt (termed RPS12FE-200A for convenience) were generated as described in Materials and Methods. Stability of RPS12FE-200A was compared with that of RPS12FE RNAs with or without a 20-A tail in an in vitro turnover assay. In the experiment shown in Fig. 6A, RPS12FE had a half-life of ~15 min, RPS12FE-20A had a half-life longer than 60 min, and RPS12-200A degraded with a half-life of ~35 min. Over several experiments, we observed that RPS12FE-200A always degraded slower than nontailed RPS12FE but more rapidly than RPS12FE-20A. Therefore, the long poly(A) tail apparently has a moderate effect on the stability of edited mitochondrial RNAs in vitro.

Poly(A) tails of mRNAs in *T. brucei* mitochondria contain interspersed uridine residues (11, 39). To determine how uridine-containing poly(A) tails affect turnover of edited RNAs, we generated RPS12FE RNA with a 3′ extension of mixed adenosine and uridine residues. The sequence of this AU tail was derived from the in vivo tail of a partially edited RPS12 RNA (35). Time course experiments were performed, showing that RPS12FE-20AU degraded at a rate similar to that of RPS12FE-20A (Fig. 6B). Over several experiments, we observed that the half-life of RPS12-20AU RNA was 1.1-fold ± 0.2-fold (n = 3) that of RPS12-20A RNA, demonstrating that a uridine-containing poly(A) tail impedes decay of edited mitochondrial RNAs to a similar extent as an adenosine homopolymer tail.

Role of poly(A) tail length and sequence in mediating turnover of edited RNAs. To further understand the turnover mechanism of edited RNAs in *T. brucei* mitochondria, we investigated the length and sequence specificity of a 3′ extension required to mediate slow decay of edited RNAs. We previously showed that, for unedited RPS12 RNA, a poly(A) tail as short as 5 nt can suboptimally stimulate decay (35). Therefore, we tested whether a poly(A)$_5$ tail is sufficient to impede decay of RPS12FE. In the experiment presented in Fig. 7, RPS12FE-5A degraded with a half-life of ~15 min, which is similar to that of nontailed RPS12FE RNAs. Data from several experi-

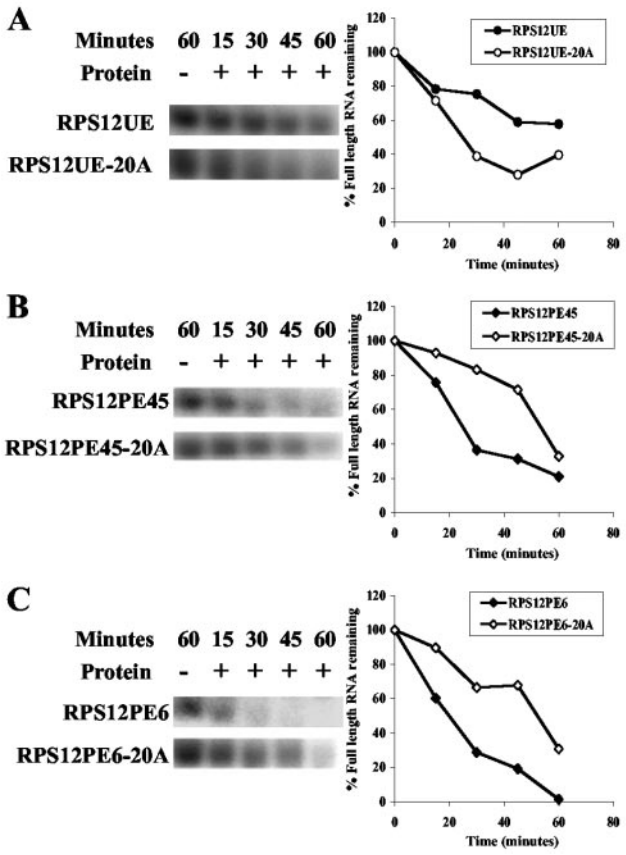

FIG. 5. Presence of edited *cis*-acting sequences encompassing six editing sites is sufficient to switch a poly(A)$_{20}$ tail from a destabilizing to a stabilizing element for RPS12 transcripts. RPS12UE (A), RPS12PE45 (B), and RPS12PE6 (C) either with or without a 20-A tail were transcribed and internally labeled in vitro. Degradation of these RNAs was measured in vitro over a time course as described in the legend for Fig. 2. Products were resolved on a 7 M urea–6% acrylamide gel and visualized by autoradiography (left panels). Percent full-length RNA remaining was determined by densitometry and was plotted for each time point in the right panels.

ments showed that the half-life of RPS12FE-5A RNA is 1.2-fold ± 0.1-fold (n = 3) that of nontailed RPS12FE RNA. Therefore, we concluded that a 5-A tail is insufficient to mediate slow turnover of edited RPS12 RNA.

Next, we asked whether homopolymer 3′ tails of nucleotide composition other than adenosine are capable of mediating slow decay of edited RNAs. That is, can any single-stranded 3′ extension impede edited RNA decay or is there a requirement for some percentage of adenosine residues? Degradation of RPS12FE RNAs containing 3′ 20-uridine tails were measured over a time course, and results were compared to those with RPS12FE-20A RNAs. As shown in Fig. 7, RPS12FE-20U degraded at a rate similar to that of RPS12FE RNAs. Over several experiments, we determined that the half-life of RPS12FE-20U RNA is 1.1-fold ± 0.2-fold (n = 3) compared to that of nontailed RPS12FE RNA. These results demonstrate that both tail length and nucleotide composition are important to poly(A)$_{20}$ tail-mediated stabilization of edited mitochondrial RNAs.

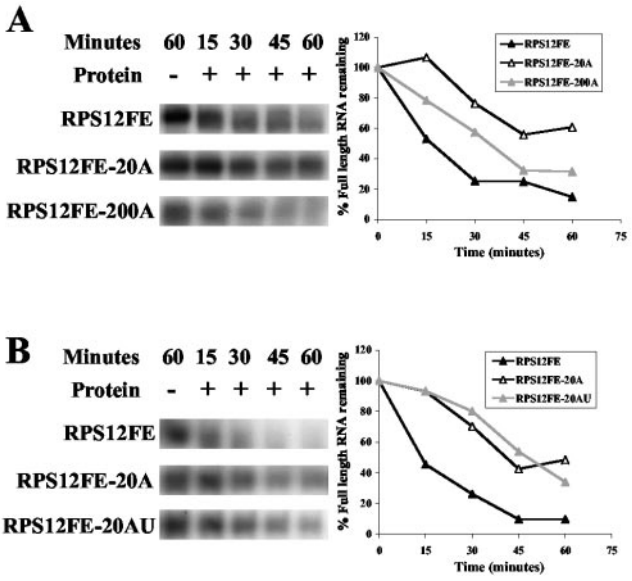

FIG. 6. Effects of two in vivo tails on stability of fully edited RPS12 RNAs. RPS12FE RNAs with no tail, a poly(A)$_{20}$ tail, a poly(A)$_{200}$ tail (A), or a poly(AU)$_{20}$ tail (B) were synthesized in vitro. These RNAs were incubated with partially purified mitochondrial fractions at 27°C for the indicated times. Products were resolved on a 7 M urea–6% acrylamide gel and visualized by autoradiography (left panels). Percent full-length RNA remaining was determined by densitometry and was plotted for each time point (right panels).

RPS12FE-20A decay is not stimulated by the addition of poly(A) to the in vitro RNA degradation assay. It has been reported in eukaryotic in vitro RNA turnover systems that addition of poly(A) homopolymer destabilizes polyadenylated RNAs. This effect is presumably achieved through sequestering the poly(A)-binding proteins that protect RNAs from nuclease digestion by binding their poly(A) tails (1, 15, 32, 43). To test whether poly(A)-binding proteins are involved in stabilizing RPS12FE-20A transcripts, poly(A) homopolymers were titrated into in vitro turnover assays, and reactions were stopped after 60 min. For all concentrations of poly(A) tested, which

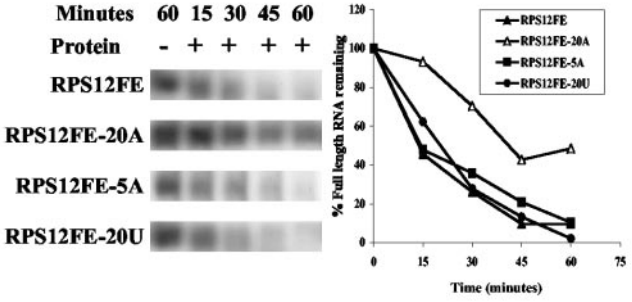

FIG. 7. A poly(A)$_5$ tail and a poly(U)$_{20}$ tail fail to stabilize fully edited RPS12 RNA. RPS12FE RNAs with either no tail or a 20-A, 5-A, or 20-U tail were transcribed and internally labeled in vitro. These RNAs were incubated with partially purified mitochondrial fractions at 27°C for the indicated times. Products were resolved on a 7 M urea–6% acrylamide gel and visualized by autoradiography (left panel). Percent full-length RNA remaining was determined by densitometry and was plotted for each time point (right panel).

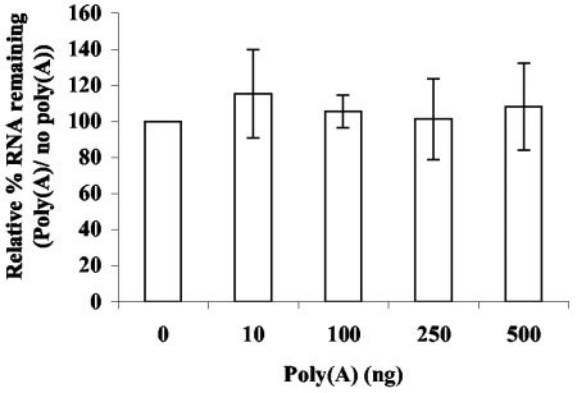

FIG. 8. Poly(A) homopolymers fail to stimulate decay of RPS12FE-20A. RPS12FE-20A RNAs were incubated with partially purified mitochondrial fractions in the presence of 0, 10, 100, 250, or 500 ng of poly(A) homopolymers at 27°C for 60 min. Products were resolved on a 7 M urea–6% acrylamide gel and visualized by autoradiography, and percent full-length RNA remaining was determined by densitometry for each poly(A) concentration. Relative percent full-length RNA remaining with poly(A) addition was calculated by using the 0-ng poly(A) reaction mixture as 100%.

encompass the concentration range required to stimulate nuclease activity in other systems (15, 32, 43), the stability of RPS12FE-20A remained largely unchanged compared to reactions performed in the absence of poly(A) (Fig. 8), strongly suggesting that poly(A)-binding proteins are not solely responsible for the stabilization of edited RPS12 RNA. Exogenous poly(A) homopolymers also had no effect on the stability of nontailed RPS12FE RNAs (data not shown). These results further support our model that the protein or protein complex that stabilizes RPS12FE-20A RNA binds not only to the poly(A) tail but also recognizes the edited *cis*-acting element.

Ribonucleolytic activities degrading fully edited RPS12 RNAs in partially purified mitochondrial extracts. To further characterize the ribonucleolytic activities involved in degradation of edited RNAs, we monitored turnover of 5′- or 3′-labeled RPS12FE RNAs with or without a 20-A tail over a time course. Degradation of both 5′-labeled RPS12FE and RPS12FE-20A led to the production of a small amount of two major intermediate products of ~275 and ~150 nt (Fig. 9A, left panel, X and Y, respectively). Because they were visualized with 5′-labeled RNA, these products must have resulted from either endonucleolytic cleavage or 3′-to-5′ exonuclease pausing. Degradation of 3′-labeled RPS12FE-20A also resulted in accumulation of two major intermediate products, in this case with sizes of ~70 and ~195 nt (Fig. 9A, right panel, X′ and Y′, respectively). In contrast, degradation of 3′-labeled RPS12FE RNA lacking a poly(A) tail resulted only in disappearance of the starting material, without the generation of any clear intermediates (Fig. 9A, right panel). The sizes of the products formed with 3′-labeled RPS12FE-20A suggest that they are the corresponding endonucleolytic counterparts of the two major degradation intermediates observed with 5′-labeled RNAs. Analysis of 3′-labeled RNAs with a 3′ phosphate group (as opposed to the 3′ OH group present on the RNAs in Fig. 9) also indicated the action of an endonuclease. In these experiments, the abundance of the ~70 and ~195 nt intermediates was increased with 3′-labeled RPS12FE-20A RNA containing a 3′ phosphate, and the corresponding products (of ~50 and ~175 nt) became visible in reactions with RPS12FE when the RNA contained a 3′ phosphate (data not shown). Presumably, the 3′ phosphate containing RNAs were protected from 3′ to 5′ exonuclease activity (see below), leading to increased accumulation of 3′ endonuclease cleavage products. The observed patterns of RPS12FE RNA decay suggest that both 20A-tailed and nontailed RNAs are subject to a modest level of endonucleolytic cleavage at least two sites. Because the resulting 5′ products are relatively stable, this suggests that the cleavage sites may correspond to regions at the 3′ ends of stem-loop structures, such that the products are resistant to 3′-to-5′ exonuclease action.

We also analyzed the decay products of 3′-labeled RNAs by TLC. As shown in Fig. 9B, turnover of both 3′-labeled RPS12FE and RPS12FE-20 resulted in the production of AMP. This indicates that, in addition to endonuclease cleavage, both RNAs are subject to degradation by a hydrolytic 3′-to-5′ exoribonuclease. Importantly, the absence of any visible intermediates by gel electrophoresis during the decay of 3′-labeled nontailed RPS12FE suggests that the 3′ products of endonuclease cleavage are very efficiently degraded by the 3′-to-5′ exonuclease (Fig. 9A, right panel). Conversely, accumulation of 3′ endonuclease cleavage products in degradation reactions with 20-A-tailed RPS12FE RNA (Fig. 9A, right panel) indicates that these products are more resistant to 3′-to-5′ exonuclease action. These results support our model in which the poly(A) tail in conjunction with a 3′ edited element blocks the action of a 3′-to-5′ exonuclease. Stabilization of the ~70-nt product further suggests that the stabilizing element is contained within the 3′ ~50 nt of the edited RPS12FE sequence, consistent with stabilization of minimally edited RNAs by polyadenylation (Fig. 5). Our data also indicate that the 3′-to-5′ exonuclease that acts on edited RPS12 RNA has properties similar to the enzyme that degrades unedited RPS12 RNA (35). Specifically, both enzymes are hydrolytic in nature (producing AMP) and slowed by a 3′ phosphate group (data not shown). Taken together, these results demonstrate that both 20-A-tailed and nontailed RPS12FE RNA are subject to endo- and exoribonuclease cleavage and that action of the 3′-to-5′ exonuclease is impeded by the presence of the 20-A tail.

DISCUSSION

In this study, we utilized the in vitro RNA turnover system previously developed in our laboratory (35) to examine the decay of edited RNAs in *T. brucei* mitochondria. We first determined the effect of edited *cis*-acting sequences on the stability of nonpolyadenylated RNAs. These studies showed that fully edited RNAs degrade at a significantly faster rate than their unedited counterparts. We also examined decay of a partially edited RNA, RPS12PE6, which is edited at only six editing sites (~10% edited). Surprisingly, this minimally edited RNA degrades at the same rapid rate as the corresponding fully edited RNA, thereby defining a small *cis*-acting element that facilitates rapid RNA decay. Since the sequences of unedited RPS12 and RPS12PE6 RNAs differ only at their very-3′ ends, these data also suggest that the mitochondrial degradation machinery recognizes edited RNA substrates from their 3′ termini, as we have previously demonstrated for unedited

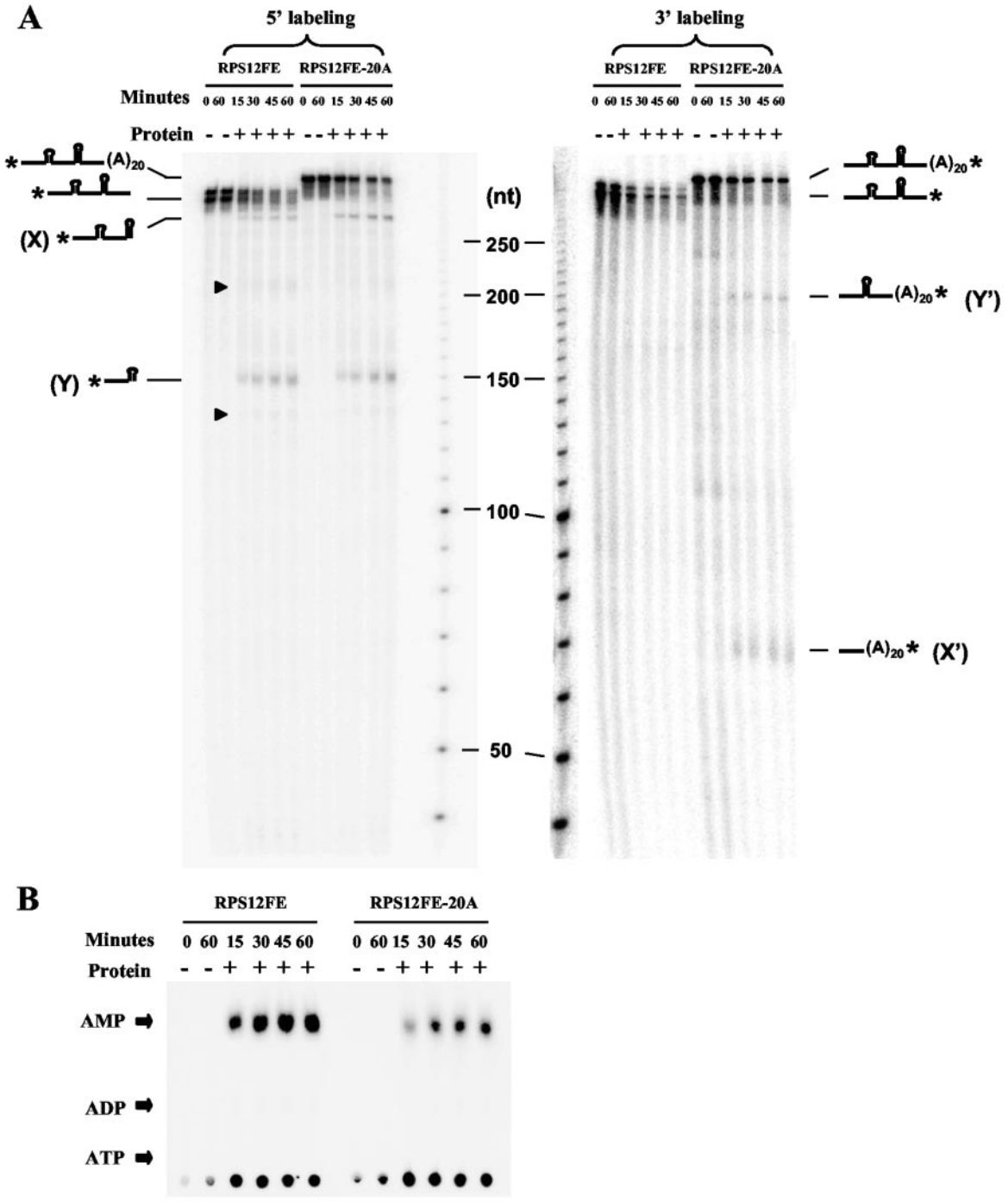

FIG. 9. Degradation of fully edited RPS12 transcripts involves both endonuclease and 3′-to-5′ exoribonuclease activities. (A) RPS12FE RNAs with or without a poly(A)$_{20}$ tail were labeled at their 5′ or 3′ ends and incubated with partially purified mitochondrial fractions for 15, 30, 45, or 60 min. Products were resolved on a 7 M urea–6% acrylamide gel and visualized by phosphorimager analysis. Shown on either side of the gel is the schematic representation of RNA molecules, both full-length substrates and proposed degradation intermediates. Corresponding endonucleolytic cleavage products are represented as X and Y (5′ products) and X′ and Y′ (3′ products). Arrowheads indicate minor degradation intermediates. (B) Products from the in vitro degradation assays using 3′-labeled RNAs as substrates were spotted onto a polyethyleneimine–F-cellulose plate, developed using 0.75 M Tris and 0.45 M HCl, and visualized by phosphorimager analysis. The migration positions of unlabeled 5′-AMP, ADP, and ATP standards were visualized by UV shadowing and are indicated on the left.

RNAs (35). Consistent with this, we showed that a 3′-to-5′ exonuclease is involved in the degradation of edited transcripts, in addition to modest endonucleolytic activities.

In vivo, edited mitochondrial RNAs contain either short (~20-nt) or long (~120- to 200-nt) poly(A) tails. Therefore, we also examined whether polyadenylation regulates the stability of edited RNAs. Previously, we reported that poly(A)$_{20}$ tails stimulate decay of unedited RPS12 and A6 RNAs, as well as

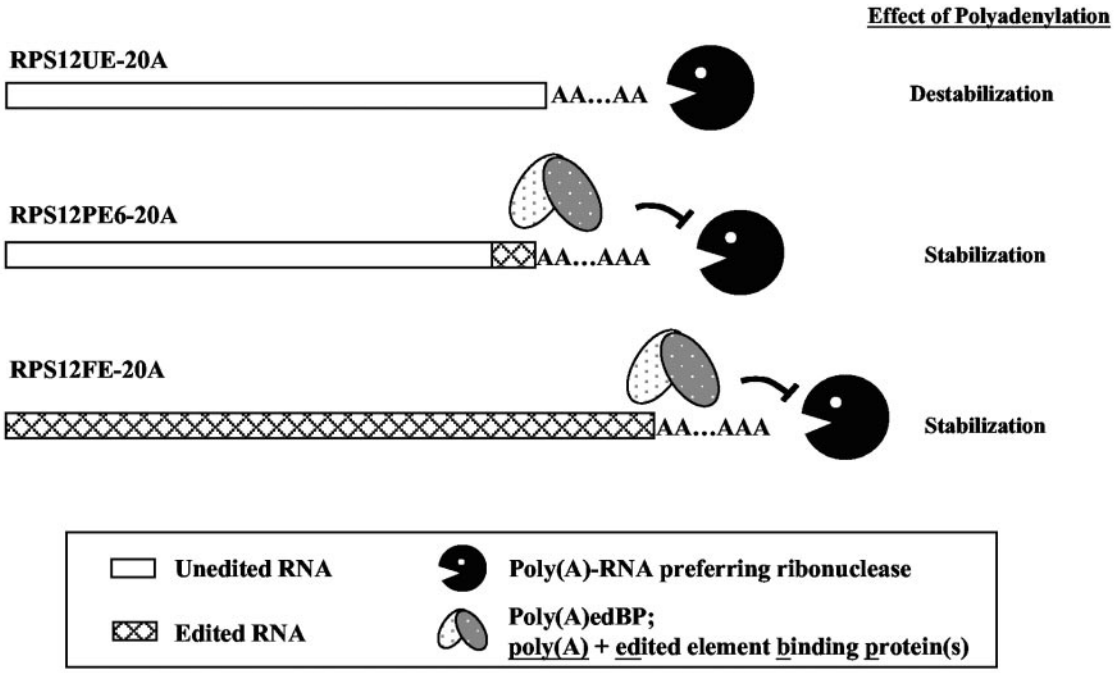

FIG. 10. Model for the role of polyadenylation in the turnover of unedited and edited RPS12 RNAs in *T. brucei* mitochondria (see text for details).

both nuclear- and plasmid-encoded transcripts (35). These data indicate that poly(A)$_{20}$ itself is sufficient to serve as a destabilizing element in this in vitro system. Intriguingly, we show here that the same poly(A)$_{20}$ tail increases the stability of edited RNAs. The stabilization of edited RNAs by a 3′ poly(A) tail was effective on both fully edited RNA and RNA edited at just six editing sites. We also showed that both the length and nucleotide composition of the 3′ tail are important for this stabilization. Taken together, our results demonstrate that the presence of a very small *cis*-acting edited sequence switches a poly(A)$_{20}$ tail from a destabilizing to a stabilizing element.

Based on results in this study and previous work (35), we developed a model for the regulation of RPS12 mRNA decay in *T. brucei* mitochondria (Fig. 10). Because a 3′ poly(A) tail is sufficient to stimulate rapid decay of both mitochondrial and reporter RNAs (35), the simplest explanation invokes the presence of a RNase activity that preferentially degrades polyadenylated substrates. This mechanism would directly account for the rapid decay of polyadenylated unedited RPS12 RNAs. Ribonucleases that preferentially degrade polyadenylated RNAs have been reported in other systems. In *E. coli*, the phosphorolytic exoribonuclease polynucleotide phosphorylase (PNPase), a major component of the RNA degradosome, preferentially binds and degrades polyadenylated and polyuridylated RNA substrates (26). We have exhaustively searched genetically and biochemically for PNPase in *T. brucei*, and results to date suggest that PNPase is absent in this system. Another example of a poly(A)-dependent RNase is found in *Arabidopsis thaliana*. AtRrp41, a phosphorolytic RNase and exosome component, has been shown to require a single-stranded poly(A) tail to load onto its RNA substrate (9). Two Rrp41 homologues have been reported in *T. brucei* (13). However, neither contains a potential mitochondrial import sequence, and so these nucleases are unlikely to be involved in mitochondrial RNA metabolism. Because the mitochondrial activity that degrades polyadenylated unedited RNA is a hydrolytic, rather than a phosphorolytic, exoribonuclease (35), this suggests a novel type of poly(A) RNA preferring exoribonuclease in our system. We are in the process of purifying this activity from *T. brucei* mitochondria and have shown that it retains its preference for polyadenylated substrates through a subsequent anion exchange chromatography step (unpublished data). Further purification and characterization of the poly(A) RNA-preferring nuclease activity will reveal whether the affinity for polyadenylated RNAs is attributed to the nuclease itself or if this property is conferred by an associated protein.

In the face of a poly(A) RNA-preferring ribonucleolytic activity, how is stabilization of edited RPS12 RNA by a poly(A)$_{20}$ tail mediated? Several mechanisms can be envisioned. First, RNA stabilization could occur through *cis* interactions. Since RNA editing results in the net addition of 104 uridine residues to RPS12FE RNA, it is possible that the base-pairing between 3′ poly(A) tails and the internal uridine tracts makes the 3′ ends of transcripts inaccessible to the mitochondrial degradation machinery. However, the minimally edited RPS12PE6 RNA, which has a net insertion of only three uridine residues over a 29-nt stretch, is also stabilized by a poly(A)$_{20}$ tail. Therefore, stabilization through a base-pairing mechanism is unlikely. Second, stabilization could occur in *trans* through binding of poly(A)-binding proteins to the poly(A) tail, reminiscent of the RNA stability mechanism in eukaryotic cytoplasm (1, 15). However, we showed here that addition of excess poly(A) homopolymers into in vitro degradation reaction mixtures, which would be expected to titrate poly(A)-binding proteins from RNA poly(A) tails (15, 32, 43), failed to stimulate decay of RPS12FE-20A RNAs. These results suggest the involve-

ment of a protection-conferring protein or protein complex that simultaneously recognizes the poly(A) tail and the small *cis*-acting edited element (Fig. 10). This protein(s), which we term poly(A)edBP (poly(A) plus edited element binding protein), would bind and protect the 3′ end of polyadenylated edited RNA from attack of the poly(A) RNA preferring nuclease activity. Accumulation of the 3′ ~70-nt endonucleolytic cleavage product (X′ in Fig. 9A), consisting of the 3′ end sequence of edited RNA and a 20-A tail, directly supports this model. Poly(A)edBP may be a single protein with affinity for both poly(A) and the small edited sequence or, as depicted in Fig. 10, this activity could be mediated by a complex comprised of two or more proteins. The ability of poly(A)edBP to block 3′-to-5′ RNA decay may result from its having a higher affinity for its substrate than does the poly(A) RNA-preferring nuclease, thereby preventing nuclease loading. Alternatively, the putative poly(A)edBP might be an abundant protein that has a high concentration in the microenvironment and so competes efficiently with the nuclease for RNA binding. It should also be noted that the same minimal *cis*-acting edited sequence that switches the poly(A) tail from a destabilizing to a stabilizing element acts to dramatically destabilize nonadenylated RPS12 RNA. This could be explained if the poly(A)edBP component that recognizes the edited element recruits the degradation machinery in the absence of a poly(A) tail. We are currently utilizing biochemical means to identify proteins that interact specifically with RPS12PE6 and RPS12PE6-A20 RNAs. Isolation and genetic manipulation of these factors will permit us to analyze the role of polyadenylation in unedited and edited RPS212 RNA turnover in vivo.

To date, our studies on the role of polyadenylation in edited RNA decay have been limited to RPS12 RNA. It will be of interest to determine how widespread this mechanism of gene regulation is in *T. brucei* mitochondria. Our results suggest that a protein involved in stabilizing edited RPS12RNA must be highly sequence specific, since its edited target sequence differs from the corresponding unedited sequence by only a few nucleotides. Since editing of different RNAs results in different edited sequences, we postulate that multiple sequence-specific poly(A)edBPs exist either for classes of transcripts or for each transcript. There is ample precedence for an RNA decay regulatory mechanism involving multiple, RNA-specific stability factors. Indeed, the majority of chloroplast mRNAs require sequence-specific nuclease-blocking *trans*-acting factors in order to accumulate (5). mRNA-specific stabilizing factors have also been reported in yeast mitochondria (10, 12).

In *T. brucei*, the abundance of specific mitochondrial RNAs is highly regulated and correlated with the modulation of mitochondrial activity throughout the life cycle. Our data suggest that both unedited and edited RNAs could serve as targets for gene regulation. A previous oligo(dT) selection assay and Northern blot analysis showed that a significant population (~42%) of steady-state unedited RPS12 RNAs are nonadenylated (30). Our finding that a poly(A) tail facilitates decay of unedited RPS12 RNA in vitro indicates that polyadenylation might be a mechanism by which cells rapidly destabilize unedited transcripts when necessary. On the other hand, in vivo, the majority of fully and partially edited RPS12 RNAs in the steady-state population are polyadenylated (30, 33). According to our results, poly(A) tails stabilize both fully and partially edited RPS12 RNAs. From an energetic standpoint, it is presumably efficient for the cell to stabilize RNAs that have begun the editing process, so that they can go on to be completely edited and translated. In addition, our data imply that in vivo, when downregulation of a certain mitochondrial gene is necessary, cells could achieve this by deadenylation of both fully and partially edited RNAs. Once deadenylated, edited RPS12 RNAs would be expected to degrade very rapidly, based on the presence of the *cis*-acting edited element that facilitates the decay of even minimally edited RNAs. In addition, turnover of edited RPS12 RNAs could also be mediated by controlling the abundance or posttranslational modification of the putative poly(A)edBP. For example, poly(A)edBP may be more abundant in the bloodstream form than in procyclic form trypanosomes, thereby accounting for the upregulation of edited RPS12 RNA in the bloodstream life cycle stage (33).

In summary, this study constitutes the first report of opposing roles for polyadenylation in regulating stability of different RNAs within a single organelle. Our studies to date have been limited to a relatively complex in vitro system. A major future goal is to understand the in vivo importance of the findings reported here. We are presently attempting to identify proteins involved in the turnover pathways of unedited and edited mitochondrial RNAs. Identification of the ribonucleases and RNA-binding proteins involved will permit future in vivo analyses of this novel mechanism for differential gene regulation.

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