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Coordination of kRNA editing and polyadenylation in *Trypanosoma brucei* mitochondria: complete editing is not required for long poly(A) tract addition

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ABSTRACT

Mitochondrial RNAs in *Trypanosoma brucei* are post-transcriptionally modified by the addition and deletion of uridylate residues in a process called kRNA editing. Unedited, partially edited and fully edited RNAs exist in the steady-state RNA population. Previous experiments have demonstrated that *T.brucei* mitochondrial RNAs contain both short (~20 nt) and long (120–200 nt) poly(A) tracts. However, it is unknown exactly what poly(A) tract lengths are present on unedited, partially edited and fully edited RNAs. To gain insight into the role of the poly(A) tract in *T.brucei* mitochondria, ribosomal protein S12 (RPS12) RNAs with short and long poly(A) tracts were purified by hybrid selection and analyzed by RT–PCR and DNA sequencing. Unedited RPS12 RNAs were found almost exclusively in populations with short poly(A) tracts. Both partially and fully edited RPS12 RNAs were found in populations with short and long poly(A) tracts. Therefore, there is a correlation between the presence of editing and the presence of the long poly(A) tract. Since a proportion of partially edited RPS12 RNAs contain long poly(A) tracts, it is unlikely that the long poly(A) tract is the sole signal for translation. Other implications for the role of polyadenylation in mitochondrial gene regulation are discussed.

INTRODUCTION

The mitochondrial genome of *Trypanosoma brucei* contains genes for rRNAs, a ribosomal protein and proteins necessary for oxidative phosphorylation (for a review see 1). The majority of the mitochondrial protein coding genes are non-functional as encoded in the mitochondrial DNA. RNAs encoded by these genes lack start codons, stop codons and/or intact open reading frames. These RNAs are modified post-transcriptionally by insertion and deletion of uridylate residues in a process called kinetoplastid RNA (kRNA) editing (for a review see 2). kRNA editing is a maturation process for the RNA as it creates start and stop codons and, in many cases, even complete open reading frames.

kRNA editing results in a complex steady-state population of RNAs. RNAs that do not require editing for maturation are termed never edited RNAs. RNAs that require editing for maturation are termed edited RNAs. Edited RNAs can be divided into three groups. RNAs that have not yet been edited are termed unedited RNAs. RNAs that have not been completely edited are termed partially edited RNAs. These RNAs are edited at their 3′-ends but not edited at their 5′-ends since editing occurs in the 3′→5′ direction (3–5). RNAs that have been completely edited are termed fully edited RNAs. It has been assumed that only never edited and fully edited RNAs are translated because they are the only RNAs with functional open reading frames, although there is little evidence for this assumption. Indeed, it is unknown if or how protein synthesis is regulated in the mitochondrion of *T.brucei*.

Northern blot analysis of *T.brucei* mitochondrial RNAs has demonstrated that many of the RNAs are present in two populations containing both short (~20 nt) and long (120–200 nt) poly(A) tracts (6,7). A similar phenomenon has been demonstrated for mitochondrial RNAs of the related organisms *Trypanosoma congolense* (8) and *Leishmania tarentolae* (9). The presence of short and long poly(A) tracts is not restricted to edited RNAs as most never edited RNAs also display two distinct poly(A) tract lengths (6). The phenomenon of differential poly(A) tract length is particularly complex for edited RNAs. Probes used in northern blot experiments that are complementary to unedited RNA sequences hybridized to RNAs that contained only short poly(A) tracts (7). Probes complementary to edited RNA sequences hybridized to both short and long poly(A) tract containing RNAs (6–9). Therefore, there appears to be a correlation between the presence of kRNA editing and the presence of the long poly(A) tract. However, it is unknown exactly what poly(A) tract size unedited, partially edited and fully edited RNAs contain, since the unedited and edited probes used in these studies could also hybridize to partially edited RNAs. Therefore, while the long poly(A) tract is unlikely to be present on unedited RNAs, it may be present on partially edited RNAs, fully edited RNAs or both.

The poly(A) tract has been shown to play a role both in translation and RNA stability in many systems (for reviews see 10–12). The longer the poly(A) tract of eukaryotic cytosolic RNAs, the greater the translational efficiency of the RNA (13–16). In the eukaryotic cytoplasm, polyadenylation also

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increases the stability of many RNAs (for reviews see 11,12). Conversely, in bacteria and chloroplasts, polyadenylated RNAs are targeted for rapid degradation (17–21). Therefore, it is possible that protein synthesis in *T.brucei* mitochondria is regulated by RNA polyadenylation and more specifically by the length of the poly(A) tract. In support of the hypothesis that polyadenylation has an essential role in the mitochondrion of *T.brucei*, the length of the poly(A) tracts of many RNAs is developmentally regulated (6–8). There are many ways in which the poly(A) tract length could regulate protein synthesis. One attractive hypothesis is that the long poly(A) tract is a signal for translation as in the eukaryotic cytoplasm. Under this circumstance, it would be expected that only never edited and fully edited mRNAs contain the long poly(A) tract since they are the only functional RNAs. Alternatively, poly(A) tract length could regulate the stability of RNAs and indirectly influence translation by altering the availability of translatable RNAs. A role of the poly(A) tract in processes other than translation and RNA stability is also possible.

To gain insight into the role of polyadenylation in *T.brucei* mitochondria, ribosomal protein S12 (RPS12) RNAs that differ in poly(A) tract length were purified by hybrid selection. The identity of these RNAs was determined by RT–PCR and DNA sequencing. Unedited RPS12 RNAs were found almost exclusively in populations that contain short poly(A) tracts. Partially edited and fully edited RPS12 RNAs were found in populations that contain both short and long poly(A) tracts. Therefore, there is a correlation between the presence of kRNA editing and the presence of a long poly(A) tract. Moreover, since a proportion of partially edited RNAs contain long poly(A) tracts, it is unlikely that the long poly(A) tract is the sole signal for translation.

MATERIALS AND METHODS

Cell culture, mitochondria isolation and RNA isolation

The procyclic form *T.brucei brucei* clone IsTaR1 from stock EATRO 164 (22) was cultured as previously described (23). Mitochondria were purified by the method of Harris *et al*. (24). Total mitochondrial RNA was prepared by the acid guanidinium/phenol/chloroform method (25); however, the mitochondria were run through a 26 gauge needle twice after lysis. Oligo(dT) selected and oligo(dT) non-selected RNA was prepared using Dynabeads oligo(dT)$_{25}$ beads (Dynal) according to the manufacturer's instructions.

Oligodeoxynucleotides

The following oligodeoxynucleotides were used in this study. The CR6-5′biotin oligodeoxynucleotide was synthesized at Integrated DNA Technologies Inc. and contained a single biotin group covalently attached to the 5′-most base. Restriction enzyme recognition sequences are underlined.

CR6-5′biotin, 5′-ACTTTAGTTTGTTATCAAAAGTGTATTAG-3′;
CR6-13, 5′-CGGGATCCAAAAACATATCTTAT-3′;
CR6-14, 5′-CGGAATTCCACTTTTGATAACAAACTAAAG-3′;
dT-RXS, 5′-GAGAATTCTCGAGTCGACTTTTTTTTTTTTTTTT-3′;
CR6-3′E, 5′-AAAAACATATCTTATATCTAAATCTAACTTACAATACGT-3′;
CR6-5′E, 5′-CGCTCGAGAATATATTTTGTTTTTTTTGCGTATGTG-3′;
CR6-9, 5′-GCGAATTCTAATACACTTTTGATAACAAACTAAAGTAAAAAGGCG-3′;
CR6-10, 5′-GCGGATCCTTTAAAAACATATCTTATTCTAAAATC-3′;
TbCR6-1, 5′-CGGAATTCAAACTAAAGTAAAAAGGCG-3′;
TbCR6-5, 5′-CTCTTCTAAACGATGTTTCTTTAACC-3′;
TbCR6-2, 5′-CGGAATTCAAAAAAAACAACGCAACATCCAAAC-3′;
TbCR6-6, 5′-GTTGTTGTTTACGTTTTGTTTTATTTG-3′.

Northern blot analysis

For the blot presented in Figure 2A, 0.2 µg of mitochondrial oligo(dT) selected RNA was fractionated on a 6% acrylamide–7 M urea gel and transferred to Nytran (Schleicher & Schuell) in 0.3× TBE (1× TBE is 90 mM Tris–borate, 2 mM EDTA) using a Semiphor Semi-Dry Transfer Unit (Hoefer Scientific Instruments) according to the manufacturer's instructions. The RNA was crosslinked to the Nytran filter using a UV Stratalinker 2400 (Stratagene). RPS12 RNAs were detected as previously described using radiolabeled PCR products as probes (7). The unedited RPS12 probe contained 179 bp of unedited RPS12 sequence and was synthesized by amplification of a plasmid containing the RPS12 gene using oligodeoxynucleotides TbCR6-1 and TbCR6-5. The edited RPS12 probe contained 135 bp of edited RPS12 sequence and was synthesized by amplification of a plasmid containing the fully edited RPS12 cDNA using oligodeoxynucleotides TbCR6-2 and TbCR6-6. To determine the extent to which different RPS12 RNAs bound to oligo(dT) beads (Fig. 2B), equal percentages of oligo(dT) non-selected and oligo(dT) selected RNA were fractionated on 6% acrylamide–7 M urea gels, transferred to Nytran and probed with unedited and edited RPS12 probes as described above. A typical experiment contained ~2 µg of oligo(dT) non-selected RNA and 0.04 µg of oligo(dT) selected RNA. Autoradiograms of northern blots were quantified using a BioRad GS-700 Imaging Densitometer using Molecular Analyst v.1.5 software (BioRad Laboratories).

Purification of RPS12 RNA by hybrid selection

RPS12 RNA was purified using an antisense biotinylated oligodeoxynucleotide complementary to the 5′ never edited sequence of RPS12 RNA by a modification of the procedure of Min and Zassenhaus (26). Both oligo(dT) selected and oligo(dT) non-selected RNA hybrid selections were done twice, each time from a different mitochondrial RNA preparation. Mitochondrial oligo(dT) selected RNA (between 0.5 and 5 µg) or mitochondrial oligo(dT) non-selected RNA (~130 µg) was denatured for 5 min at 90°C in 1× annealing buffer (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$) in the presence of excess CR6-5′biotin oligodeoxynucleotide. The reaction was then incubated at 37°C for 2 h to allow annealing of the oligodeoxynucleotide to the RNA. The oligodeoxynucleotide:RNA complexes were captured from solution using Dynal M-280 streptavidin beads (Dynal) following the manufacturer's instructions. Briefly, 350–500 µg of Dynal M-280 streptavidin beads in B+W buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) were added to the reaction and incubated at room temperature for 10 min with gentle rotation. The binding reaction was carried out in a final concentration of 0.5× B+W buffer. The beads were captured in a Magnetic Particle Concentrator for 30 s, washed three times at room temperature with three bead volumes of high salt wash buffer (10 mM Tris–HCl, pH 7.8, 500 mM NaCl, 5 mM EDTA) and then washed three times at room temperature with three bead volumes of low salt wash buffer (10 mM Tris–HCl, pH 7.8, 20 mM NaCl, 5 mM EDTA). The captured RNA was eluted from the oligodeoxynucleotide by heating the beads at 80°C for 10 min in 10 µl elution buffer (10 mM Tris–HCl, pH 8.0, 10 mM DTT, 1 mM

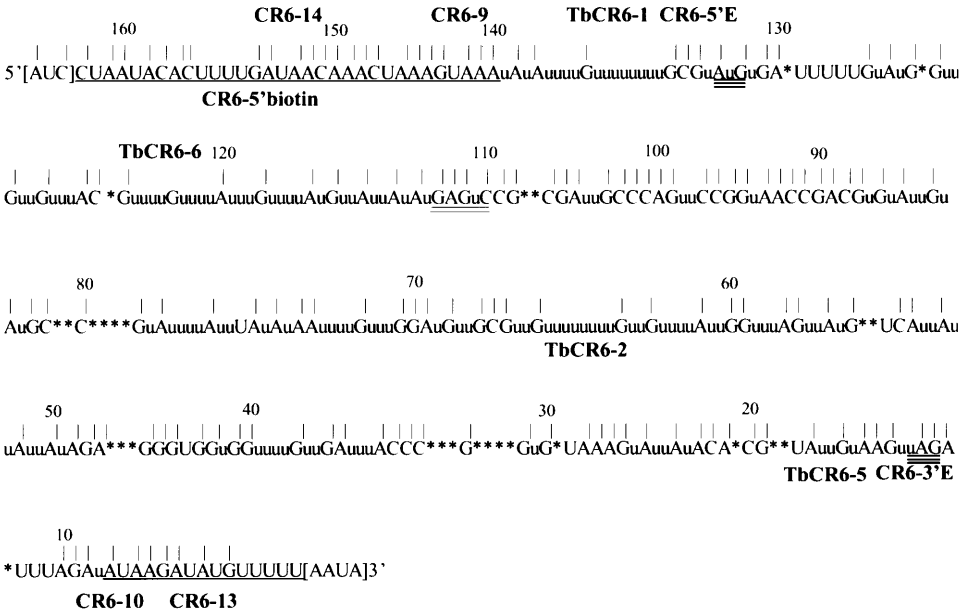

Figure 1. The *T.brucei* RPS12 RNA sequence (7). Nucleotides encoded in the mitochondrial genome are shown in upper case. Uridines added by kRNA editing are shown in lower case. Uridines encoded in the mitochondrial DNA and deleted by kRNA editing are denoted by an asterisk. Sequence shown in brackets indicates sequence present in a minority of RPS12 clones. Potential editing sites (sites between non-uridine residues) are numbered and proceed 3′→5′. 5′ and 3′ never edited regions are single underlined. A *Hin*fI restriction site (GANTC) created by kRNA editing is double underlined. Start (AUG) and stop (UAG) codons created by kRNA editing are triple underlined. Oligodeoxynucleotide names are displayed approximately above (sense orientation) or below (antisense orientation) their respective sequence in the RNA.

EDTA). The elution was repeated once and the eluates were combined. The hybrid selected RNAs were labeled at their 3′-ends with 80 µCi cytidine 3′,5′-[5′-^{32}P]bisphosphate ([^{32}P]pCp; 800 mCi/mmol; NEN Life Sciences Products) in a volume of 20 µl containing 15% glycerol, 10% DMSO, 5 mM $MgCl_2$, 25 mM HEPES, pH 8.3, 1.6 mM DTT and 0.25 mM ATP using T4 RNA ligase (Gibco BRL). This reaction was carried out overnight at 4°C. An equal volume of denaturing loading buffer (90% formamide, 1× TBE, 0.1% xylene cyanol, 0.1% bromophenol blue) was added and the sample was heated at 90°C for 3 min. The RNAs were fractionated on a 14 inch, 6% acrylamide–7 M urea gel run at 40 W. RNA Century Plus RNA size standards were used (Ambion). The gels were exposed to Kodak X-AR film at –80°C using an intensifying screen and RNAs were detected by autoradiography. Different RNA populations were excised from the gel and eluted from gel slices overnight at room temperature in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.75 M NH_4OAc. The RNAs were precipitated with isopropanol and resuspended in DEPC-treated water.

cDNA synthesis, PCR and cloning

The hybrid selected RNA subpopulations obtained as described above served as templates for cDNA synthesis using Superscript II reverse transcriptase (Gibco BRL) and dT-RXS as a primer. In some cases, the RNA was first treated with RNase H (Gibco BRL) and oligo(dT)$_{15}$ to remove poly(A) tracts (6) and cDNA was then synthesized using the CR6-13 primer. All PCR reactions contained 0.3 µM of each oligodeoxynucleotide and were performed in a Perkin Elmer GeneAmp PCR System 2400 thermal cycler. To amplify total RPS12 cDNA, CR6-14 and CR6-13 primers were used under the following conditions: 94°C for 30 s (1 cycle); 94°C for 30 s, 37°C for 30 s, 72°C for 30 s (4 cycles); 94°C for 30 s, 52°C for 30 s, 72°C for 30 s (26 cycles); 72°C for 5 min (1 cycle). To amplify fully edited RPS12 cDNA, CR6-5′E and CR6-3′E primers were used under the following conditions: 94°C for 1 min (1 cycle); 94°C for 30 s, 58°C for 30 s, 72°C for 30 s (30 cycles); 72°C for 5 min (1 cycle). To amplify unedited RPS12 cDNA, CR6-10 and CR6-9 primers were used under the following conditions: 94°C for 1 min (1 cycle); 94°C for 30 s, 37°C for 30 s, 72°C for 30 s (35 cycles); 72°C for 5 min (1 cycle). PCR products were fractionated on 1.5 or 2% agarose gels, stained with 0.5 µg/ml ethidium bromide and visualized by UV illumination. For figure preparation, photographs of ethidium bromide stained gels were analyzed on a BioRad GS-700 Imaging Densitometer using Molecular Analyst v.1.5 software (BioRad Laboratories). The contrast on scanned images was inverted. For DNA sequence analysis, PCR products were digested with *Eco*RI and *Bam*HI (Gibco BRL), ligated into the phagemid pBSCII SK– (Stratagene) and transformed into DH5α competent *Escherichia coli* cells (Gibco BRL). Recombinant colonies were identified by colony PCR or by restriction digestion analysis of purified phagemids. Phagemids were sequenced at the Center for Advanced Molecular Biology and Immunology DNA Sequencing Facility (SUNY at Buffalo, Buffalo, NY) using automated DNA sequencing technology.

RESULTS

Northern blot analysis of RPS12 RNA

The RPS12 gene (formerly called the CR6 gene) is located in the mitochondrial maxicircle DNA and the sequence of fully edited RPS12 RNA predicts a protein with homology to ribosomal

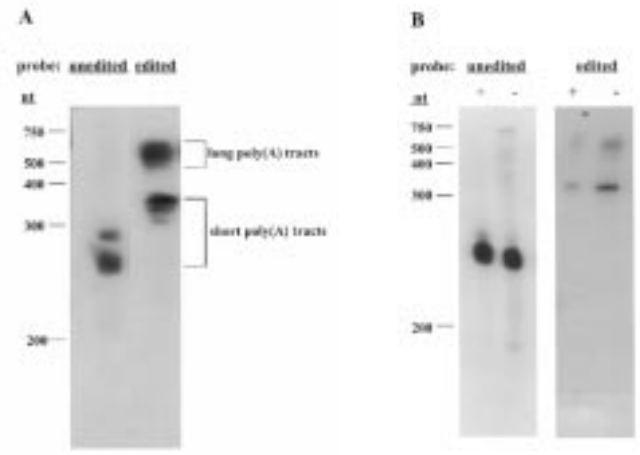

Figure 2. Analysis of RPS12 RNA by northern blotting. (**A**) *Trypanosoma brucei* mitochondrial oligo(dT) selected RNA (0.2 µg) was fractionated on a 6% acrylamide–7 M urea gel, transferred to Nytran and probed with a radiolabeled PCR product corresponding to unedited RPS12 sequence (left) or a radiolabeled PCR product corresponding to edited RPS12 sequence (right). RNA subpopulations with short and long poly(A) tracts (7) are indicated. The migration of RNA molecular size standards are displayed on the left. (**B**) Northern blot analysis of mitochondrial RNAs fractionated on oligo(dT) magnetic beads. Total mitochondrial RNA was fractionated on oligo(dT) beads and equal percentages of oligo(dT) selected RNA [RNA that bound to oligo(dT) beads; ~0.04 µg] and oligo(dT) non-selected RNA [RNA that did not bind to oligo(dT) beads; ~2 µg] were electrophoresed on a 6% acrylamide–7 M urea gel, transferred to Nytran and hybridized to the same probes as used in (A). Lanes marked (+) contained oligo(dT) selected RNAs and lanes marked (–) contained oligo(dT) non-selected RNAs. The migration of RNA molecular size standards are displayed on the left.

protein S12 (27). *Trypanosoma brucei* RPS12 RNA is edited by the addition of 132 uridines and deletion of 28 uridines (Fig. 1; 7). Exclusive of the poly(A) tract, unedited RPS12 RNA is 221 nt and fully edited RPS12 RNA is 325 nt. Editing creates the start codon, stop codon and open reading frame for this RNA. Partially edited RPS12 RNAs are abundant in the steady-state RNA population (K.Militello and L.Read, unpublished results; this report). It has been previously demonstrated by northern blot experiments that RPS12 RNAs contain both short and long poly(A) tracts (7). Typical RPS12 northern blots are shown in Figure 2A; RPS12 RNA subpopulations with short and long poly(A) tracts are indicated.

The feasibility of using mitochondrial poly(A)$^+$ RNA as the source of RPS12 RNA for subsequent experiments was determined by fractionation of total mitochondrial RNA on oligo(dT) beads. RNAs that were captured and subsequently eluted from the oligo(dT) beads were designated oligo(dT) selected RNAs. RNAs that were not captured by the oligo(dT) beads were designated oligo(dT) non-selected RNAs. Equal percentages of both oligo(dT) selected and oligo(dT) non-selected RNAs were analyzed for RPS12 RNA by northern blotting using unedited and edited RPS12 probes (Fig. 2B). The majority (58 ± 18%, n = 4) of RNAs that hybridized to the unedited RPS12 probe were found in the oligo(dT) selected RNA population, demonstrating that the majority of these RNAs contain poly(A) tracts (Fig. 2B). The remaining 42% of the RNA in the oligo(dT) non-selected population may be non-polyadenylated RPS12 RNAs as non-polyadenylated, unedited *T.brucei* mitochondrial RNAs have previously been reported (5). It is also possible that these RNAs are polyadenylated, but the oligo(dT) capture step is not 100% efficient. Surprisingly, 91 ± 6% (n = 3) of RNAs that hybridized to the edited RPS12 probe were found in the oligo(dT) non-selected RNA population (Fig. 2B). Since it has been demonstrated that these RNAs contain poly(A) tracts by digestion with RNase H and oligo(dT) (7) and it is possible to generate cDNA from these RNAs using oligo(dT) as a primer (Figs 7A and 9), it is likely that these RNAs are not captured efficiently by oligo(dT) beads. Base pairing between the poly(A) tract and uridine-rich regions added by kRNA editing may inhibit binding of the poly(A) tracts to oligo(dT) beads. Since many polyadenylated RPS12 RNAs were not captured efficiently by oligo(dT) beads, we will refer to RNAs as oligo(dT) selected or oligo(dT) non-selected instead of the standard terminology of poly(A)$^+$ and poly(A)$^-$. Based on our northern blot results, the oligo(dT) selected RNAs were the source of RNAs used to analyze the poly(A) tract length of unedited RPS12 RNAs and the oligo(dT) non-selected RNAs were the source of RNAs used to analyze the poly(A) tract lengths of both extensively edited (defined as RNAs edited past site 110; see *Hin*fI restriction site in Fig. 1) and fully edited RPS12 RNAs.

Hybrid selection of RPS12 RNA

An antisense biotinylated oligodeoxynucleotide (CR6-5′biotin) that is complementary to a region in the 5′-portion of the RNA that is never edited (Fig. 1) was used to purify RPS12 RNAs from oligo(dT) selected and oligo(dT) non-selected RNAs as described in Materials and Methods. All RPS12 RNAs have the 5′ never edited region, regardless of the extent of editing. RPS12 RNAs purified from oligo(dT) selected RNA were fractionated by gel electrophoresis into four RNA subpopulations, designated subpopulations 1–4 (Fig. 3A). We conclude these RNAs are RPS12 RNAs for the following three reasons. (i) These RNAs are the same sizes as RPS12 RNAs detected by northern blotting (7; compare Figs 3A and 2A). (ii) This pattern was not observed when biotinylated oligodeoxynucleotides antisense to apocytochrome b (28) or NADH dehydrogenase subunit 9 (29) RNAs were used (data not shown). (iii) All four subpopulations of hybrid selected RPS12 RNA could be amplified by RT–PCR using RPS12-specific primers (Figs 4 and 6).

RPS12 RNA was also purified from oligo(dT) non-selected RNA (Fig. 3B). These RNAs did not show the characteristic RPS12 pattern observed in RPS12 northern blots (7; Fig. 2A) and the RPS12 RNA hybrid selected from oligo(dT) selected RNAs (Fig. 3A). Instead, heterogeneously sized RNAs were observed which ranged from 100 to 750 nt, although subpopulations of the expected sizes could be faintly discerned. RNAs of three different sizes were purified from the gel. Subpopulation 5 was 300–375 nt which is in the range of fully edited RPS12 RNA with short poly(A) tracts. Subpopulation 6 was 450–600 nt which is in the range of fully edited RPS12 RNA with long poly(A) tracts. RNAs of 100–110 nt were also purified and designated subpopulation X. These RNAs served as a negative control population.

The poly(A) tract sizes of the purified RPS12 RNA subpopulations could not be determined directly using digestion with RNase H and oligo(dT)$_{15}$ (6) or digestion with RNase T1 (30) due to a lack of material purified from the gel. However, we conclude that subpopulations 1–3 and 5 have short poly(A) tracts since identical size RPS12 RNA populations have short poly(A) tracts

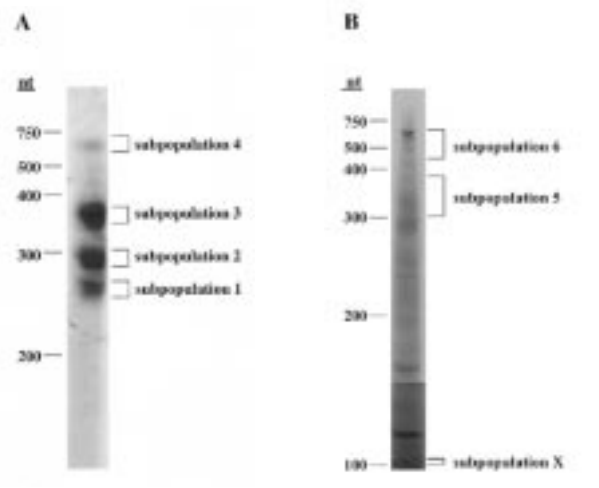

Figure 3. Purification of RPS12 RNA. RPS12 RNA was purified from either mitochondrial oligo(dT) selected RNA (**A**) or oligo(dT) non-selected RNA (**B**) using the oligodeoxynucleotide CR6-5′biotin as described in Materials and Methods. Regions of the gel from which different subpopulations of RNA were eluted are shown on the right. The migration of RNA molecular size standards are displayed on the left. In (B), the film was cut into two pieces due to size constraints of the scanner, accounting for the apparent crack at 140 nt.

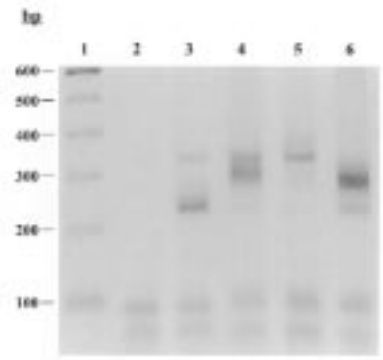

Figure 4. RT–PCR analysis of RPS12 RNA purified from oligo(dT) selected RNA. RNA subpopulations 1–4 (Fig. 3A) were analyzed for total RPS12 RNA by RT–PCR as described in Materials and Methods. Lane 1, DNA molecular size markers; lane 2, no cDNA control reaction; lane 3, subpopulation 1 cDNA; lane 4, subpopulation 2 cDNA; lane 5, subpopulation 3 cDNA; lane 6, subpopulation 4 cDNA.

as deduced from northern blot analysis of RNase H/oligo(dT) digested RNA (7). By the same criterion, long poly(A) tracts are present on RNAs from subpopulations 4 and 6.

Analysis of purified RPS12 RNAs by RT–PCR and DNA sequencing

The degree of editing of the RPS12 RNAs in the four subpopulations purified from oligo(dT) selected RNA (Fig. 3A) was determined. RNAs from subpopulations 1–4 were amplified by RT–PCR using primers that hybridize to 5′ and 3′ never edited regions of RPS12 RNA (Fig. 1) and therefore amplify all RPS12 RNAs (Fig. 4). In each case, RT–PCR resulted in products with the sizes expected for RPS12 RNAs (231–335 bp). No products were observed in control reactions without cDNA template. The increasing size of the RT–PCR products from subpopulations 1–3 was consistent with the increasing size of purified RNA (Fig. 3A). RT–PCR products from subpopulation 4 were similar to the sizes of the RT–PCR products from subpopulations 1 and 2.

The products of each of the four RT–PCR reactions were cloned into the phagemid pBSCII SK– and several randomly chosen clones were analyzed by DNA sequencing (Fig. 5). Clones from subpopulations 1, 2 and 4 were all partially edited. Twelve of 15 clones from subpopulation 3 were partially edited and the remaining three clones were unedited. Despite extensive analysis of the sequence data, we could discern no correlation between the amount of editing and the presence of the long poly(A) tract. In addition, there was no obvious correlation between the presence and/or length of the junction region, defined as incompletely edited sequences at the boundary of unedited and fully edited sequences (31) and any specific RNA subpopulation. However, since partially edited RNAs were found in all four subpopulations, we conclude that partially edited RPS12 RNAs contain both short poly(A) tracts (subpopulations 1–3) and long poly(A) tracts (subpopulation 4).

To determine the subpopulations that contained the majority of the unedited RPS12 RNA, the four RPS12 RNA subpopulations purified from oligo(dT) selected RNA were analyzed by RT–PCR using unedited RPS12-specific primers with different template concentrations (Fig. 6). The majority of the unedited RPS12 RNAs were found in subpopulation 1 (Fig. 6, lanes 3–6). The expected 239 bp RT–PCR product was generated from this subpopulation even at the lowest cDNA concentration. Unedited RPS12 RNAs were also detected in subpopulations 2–4, but only at the highest cDNA concentration, which is 50-fold higher than the cDNA concentration at which unedited RNAs were detected in subpopulation 1 (Fig. 6, lanes 10, 14 and 18). Moreover, in another experiment, unedited RPS12 RNAs were only detected in subpopulation 1 (data not shown). We therefore conclude that the vast majority of unedited RPS12 RNAs have short poly(A) tracts. Our data indicate that a very small percentage of unedited RPS12 RNAs have long poly(A) tracts. However, we cannot rule out gel artifacts as a cause for this observation and therefore unedited RPS12 RNAs may never have long poly(A) tracts.

RPS12 RNAs purified from oligo(dT) non-selected RNA (Fig. 3B) were analyzed for extensively and fully edited RPS12 RNA. cDNA was synthesized from subpopulations 5, 6 and X using either dT-RXS or CR6-13 oligodeoxynucleotide as a primer. dT-RXS oligodeoxynucleotide is a derivative of oligo(dT) (Materials and Methods) and CR6-13 oligodeoxynucleotide is able to hybridize to the 3′-ends of all RPS12 molecules (Fig. 1). The CR6-13 primer was used in the event the poly(A) tract was blocked by base pairing to uridine-rich regions in the body of the RNA. In this case, the poly(A) tract was first removed by treatment with RNase H and oligo(dT)$_{15}$ before synthesis of cDNA using the CR6-13 oligodeoxynucleotide as a primer. The purified RPS12 RNAs were amplified by RT–PCR using primers that hybridize to the 5′ and 3′ never edited regions of RPS12 RNA and therefore amplify all RPS12 RNAs. The results are shown in Figure 7A. No RPS12 RNA was detected in control reactions without cDNA (Fig. 7A, lane 2) or using subpopulation X cDNA (Fig. 7A, lanes 3 and 6) which, based on its migration, would not have been expected to contain RPS12 RNA. Products of the expected size for RPS12 RNA (231–335 bp) were observed in

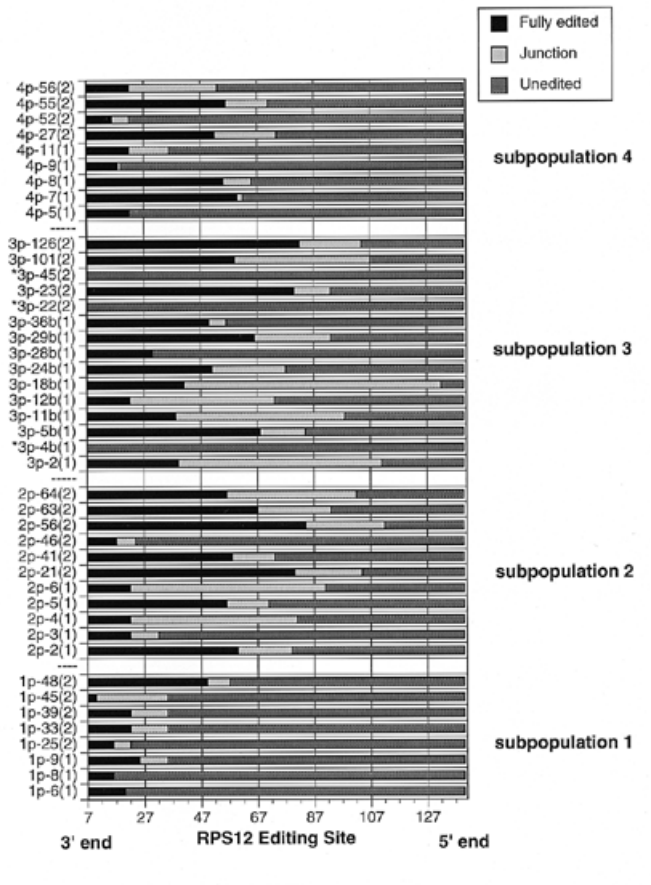

Figure 5. DNA sequence analysis of RPS12 RNAs purified from oligo(dT) selected RNA. The RT–PCR products from purified subpopulations 1–4 (Fig. 4) were cloned into the phagemid pBSCII SK– and randomly selected clones were analyzed by DNA sequencing. Individual clones are displayed 3′→5′ and editing sites are denoted below. Sites 1–7 and 141–165 are not displayed since no editing occurs at these sites *in vivo*. The first number of each clone is the RPS12 subpopulation from which it originated and the number in parentheses is the experiment number. Asterisks are placed in front of unedited clones. The black bar represents fully edited regions, the light gray bars represent junction regions and the dark gray bars represent unedited regions.

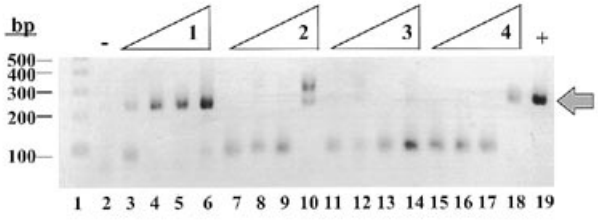

Figure 6. Detection of unedited RPS12 RNA by RT–PCR. RNA subpopulations 1–4 (Fig. 3A) were analyzed for unedited RPS12 RNA by RT–PCR as described in Materials and Methods. The arrow indicates the location of the predicted 239 bp product. Lane 1, DNA molecular size markers; lane 2, no cDNA control reaction (–); lanes 3–6, titration of subpopulation 1 cDNA; lanes 7–10, titration of subpopulation 2 cDNA; lanes 11–14, titration of subpopulation 3 cDNA; lanes 15–18, titration of subpopulation 4 cDNA; lane 19, positive control reaction using a plasmid containing the RPS12 gene as a template (+). The range of cDNA titration for each sample was 1:50, 1:10, 1:5 and 1. The identity of the larger band observed at the highest concentration of subpopulation 2 cDNA is unknown.

reactions using subpopulation 5 cDNA (Fig. 7A, lanes 4 and 7) and subpopulation 6 cDNA as template (Fig. 7A, lanes 5 and 8). The outcome of the RT–PCR was similar regardless of whether the dT-RXS or CR6-13 oligodeoxynucleotide primed cDNA was used as a template. Although these subpopulations originate from oligo(dT) non-selected RNA, they do contain poly(A) tracts as demonstrated by the ability of oligo(dT) to prime cDNA synthesis for RT–PCR analysis.

To determine if extensively edited RPS12 RNAs were present in subpopulations 5 and 6, the RT–PCR products were digested with the restriction enzyme *Hin*fI. kRNA editing creates a unique *Hin*fI site at editing site 110 (Fig. 1). If the RNA is not edited at site 110, the RT–PCR product will not be digested (Fig. 7B). If the RNA is edited at site 110, the RT–PCR product will be digested into a 219 bp 3′ fragment and a variable length 5′ fragment, ranging from 78–115 bp depending on the extent of editing 5′ to site 110. A small percentage of the subpopulation 5 RT–PCR product is digested by *Hin*fI indicating that some of the RPS12 RNAs in this subpopulation are edited at site 110 (Fig. 7B, lanes 2 and 3). It is unlikely that the restriction digestion was incomplete as these conditions allow almost complete digestion of subpopulation 6 RT–PCR products (Fig. 7B, lanes 4 and 5). Restriction digestion of subpopulation 5 RT–PCR product using a restriction enzyme that recognizes all RPS12 DNAs demonstrated that the RT–PCR product is indeed RPS12 DNA (data not shown). *Hin*fI almost completely digested subpopulation 6 RT–PCR product, indicating that the majority of the RPS12 RNAs in this subpopulation are edited at site 110 (Fig. 7B, lanes 4 and 5). We conclude that extensively edited RPS12 RNAs have both short poly(A) tracts (subpopulation 5) and long poly(A) tracts (subpopulation 6).

The sequence composition of the RPS12 RNAs in the two subpopulations purified from oligo(dT) non-selected RNA was determined. The RT–PCR products (Fig. 7A) were cloned into the phagemid pBSCII SK– and several randomly chosen clones were analyzed by DNA sequencing (Fig. 8). Eleven of 12 subpopulation 5 clones were partially edited. The remaining clone was unedited. Eight of eight subpopulation 6 clones were partially edited. The size of sequenced clones from subpopulation 5 reflected the size of the subpopulation 5 RT–PCR products. However, although the majority of the RT–PCR products from subpopulation 6 appeared to be edited at site 110 by digestion with *Hin*fI (Fig. 7B), sequence analysis of these RT–PCR products indicated that only one of eight clones [6p-4(1)] was edited to site 110. Therefore, a bias may exist that promotes cloning of less edited RT–PCR products in this sample. Again, there was no correlation between the presence and/or length of the junction region and a specific RNA subpopulation. All RNAs in subpopulation 6 were edited to some extent, indicating that the presence of editing is correlated with the presence of the long poly(A) tract. This observation is consistent with the sequence analysis of the subpopulation 4 RNAs (Fig. 5). However, it is difficult to make conclusions regarding any correlation between the amount of editing and the presence of the long poly(A) tract due to the cloning bias. Nonetheless, these data provide further evidence that partially edited RNAs contain both short poly(A) tracts (subpopulation 5) and long poly(A) tracts (subpopulation 6).

RT–PCR was used to determine the location of the fully edited RPS12 RNAs in the two subpopulations purified from oligo(dT)

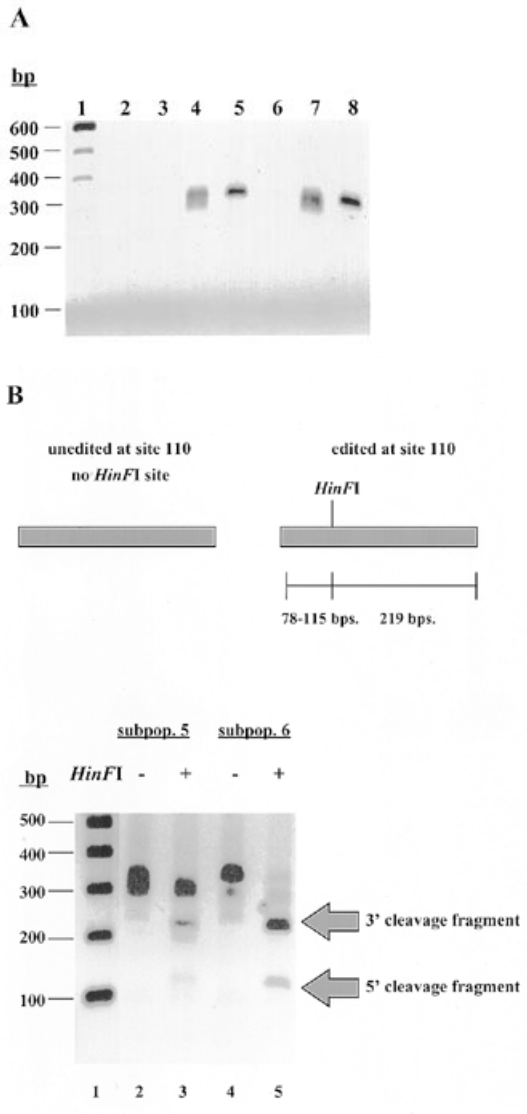

Figure 7. Detection of extensively edited RPS12 RNAs. (**A**) RT–PCR analysis. RNA subpopulations 5, 6 and X (Fig. 3B) were analyzed for total RPS12 RNA by RT–PCR as described in Materials and Methods. Lane 1, DNA molecular size markers; lane 2, no cDNA control reaction; lanes 3 and 6, subpopulation X cDNA; lanes 4 and 7, subpopulation 5 cDNA; lanes 5 and 8, subpopulation 6 cDNA. cDNA was synthesized using either the CR6-13 primer (lanes 3–5) after treatment with RNase H and oligo(dT)$_{15}$ or the dT-RXS primer (lanes 6–8). (**B**) Restriction digestion analysis. kRNA editing creates a *Hin*FI site at editing site 110. The expected sizes of the digestion products of RPS12 RT–PCR products unedited and edited at site 110 are indicated in the cartoon. Subpopulation 5 and 6 RT–PCR products (shown in Fig. 7A) were either left undigested or digested with *Hin*FI. The locations of the 5′ and 3′ cleavage fragments are indicated by arrows. Lane 1, DNA molecular size markers; lane 2, undigested subpopulation 5 RT–PCR product; lane 3, subpopulation 5 RT–PCR product digested with *Hin*FI; lane 4, undigested subpopulation 6 RT–PCR product; lane 5, subpopulation 6 RT–PCR product digested with *Hin*FI.

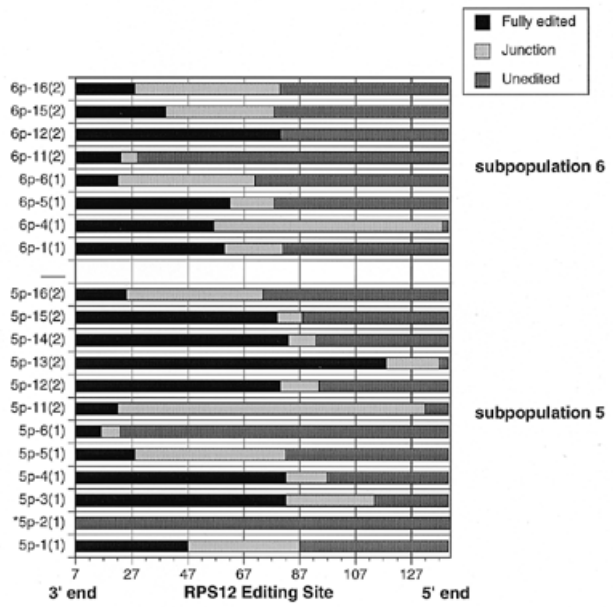

Figure 8. DNA sequence analysis of RPS12 RNAs purified from oligo(dT) non-selected RNA. The subpopulation 5 and 6 (Fig. 7A) RT–PCR products were cloned into the phagemid pBSCII SK– and randomly selected clones were chosen for DNA sequencing. Individual clones are displayed 3′→5′ and the positions of editing sites are denoted below. Sites 1–7 and 141–165 are not displayed since no editing occurs at these sites *in vivo*. The first number of each clone is the RPS12 subpopulation from which it originated and the number in parentheses is the experiment number. Asterisks are placed in front of unedited clones. The black bar represents fully edited regions, the light gray bars represent junction regions and the dark gray bars represent unedited regions.

non-selected RNA (Fig. 9). Primers specific to fully edited RPS12 RNA were used with varying concentrations of cDNA template. No fully edited RT–PCR product was generated using subpopulation X cDNA (data not shown) or in a reaction containing no cDNA template (Fig. 9, lane 2). A 302 bp fully edited RT–PCR product was generated using both subpopulation 5 cDNA (Fig. 9, lanes 3–6 and 11–14) and subpopulation 6 cDNA (Fig. 9, lanes 7–10 and 15–18). Again, the outcome of the RT–PCR was similar regardless of whether dT-RXS or CR6-13 oligodeoxynucleotide primed cDNA was used. We conclude that fully edited RPS12 RNAs contain both short poly(A) tracts (subpopulation 5) and long poly(A) tracts (subpopulation 6). The ratio of fully edited RPS12 RNAs containing short and long poly(A) tracts varied between experiments (data not shown).

DISCUSSION

Trypanosoma brucei mitochondrial RNAs are present in populations containing both short and long poly(A) tracts and poly(A) tract lengths are often developmentally regulated (6–9). However, because of the complexity added to the mitochondrial RNA population by kRNA editing, previous northern blot experiments have not been able to distinguish precisely which sequence classes of a given RNA (unedited, partially edited or fully edited) contain which poly(A) tract lengths. RPS12 RNAs were purified by hybrid selection and separated by gel electrophoresis into populations that differ in poly(A) tract length. RT–PCR and DNA sequencing analysis of the hybrid selected RNAs allowed us to determine the sequence classes of RPS12 RNAs present in these populations and therefore to determine the relationship between the presence of kRNA editing and poly(A) tract length. Unedited RPS12 RNAs were found almost exclusively in subpopulations that contain short poly(A) tracts (Fig. 6). These results are consistent with northern blot experiments demonstrating that

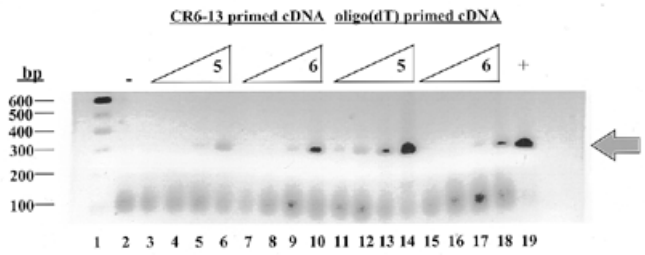

Figure 9. Detection of fully edited RPS12 RNA by RT–PCR. RNA subpopulations 5 and 6 were analyzed for fully edited RPS12 RNA by RT–PCR as described in Materials and Methods. The arrow indicates the location of the predicted 302 bp product. Lane 1, DNA molecular size markers; lane 2, no cDNA control reaction (–); lanes 3–6 and 11–14, titration of subpopulation 5 cDNA; lanes 7–10 and 15–18, titration of subpopulation 6 cDNA; lane 19, positive control reaction using a plasmid containing the fully edited RPS12 cDNA as a template (+). The range of cDNA titration for each sample was 1:100, 1:50, 1:10 and 1. cDNA was synthesized using either the CR6-13 oligodeoxynucleotide after treatment with RNase H and oligo(dT)$_{15}$ (lanes 3–10) or the dT-RXS oligodeoxynucleotide (lanes 11–18).

RNAs hybridizing to unedited probes contain short poly(A) tracts (7). Partially edited and fully edited RPS12 RNAs were found in populations that contain both short and long poly(A) tracts (Figs 5, 8 and 9). These results are consistent with northern blot experiments in which probes complementary to edited RNA sequences detected RNAs with both poly(A) tract lengths (6–9).

In many systems, an increase in the length of the poly(A) tract is correlated with an increase in the translation of a specific RNA (for a review see 10,13–16). We originally speculated that the long poly(A) tract might be a signal for translation in the mitochondrion of *T.brucei* and could provide a mechanism by which only translatable RNAs associate with ribosomes. In this system, only never edited and fully edited RNAs are predicted to be translated since they are the only RNAs with complete open reading frames. Thus, this hypothesis predicts that only fully and never edited RNAs would possess long poly(A) tracts. However, the present study indicates that some partially edited RPS12 RNAs contain long poly(A) tracts. Therefore, the long poly(A) tract is unlikely to provide a mechanism by which only mature RNAs are translated in *T.brucei* mitochondria. It cannot be ruled out, however, that long poly(A) tracts are required for translation, but that an additional signal present only in fully and never edited RNAs is also required. The establishment of an *in vitro T.brucei* mitochondrial translation system will be necessary to test these hypotheses.

As an alternative to, or in addition to, a role in translation, polyadenylation may regulate RNA stability in the mitochondrion of *T.brucei*. The two poly(A) tract sizes could provide differential stability to different subpopulations of mitochondrial RNAs. One mechanism by which this may be accomplished is suggested by northern blot data of oligo(dT) selected and oligo(dT) non-selected mitochondrial RNA (Fig. 2A). A significant proportion of extensively and fully edited RPS12 RNAs were unable to bind to oligo(dT) beads even though RT–PCR experiments demonstrated that these RNAs contained poly(A) tracts. We speculate that base pairing occurs between the poly(A) tract and uridine-rich regions added by kRNA editing and that this base pairing blocks association of the poly(A) tract with oligo(dT) beads. If this RNA structure exists *in vivo*, it could regulate the stability of uridine-rich RNAs. Double-stranded RNA structures are likely to be more resistant to nuclease digestion. The poly(A) tract could also regulate RNA stability through interactions with specific proteins such as poly(A)-binding proteins (for a review see 11) or poly(A) nucleases (32). Pulse–chase RNA stability experiments will be necessary to analyze the role of the poly(A) tract length in the stability of RNAs in *T.brucei* mitochondria and these experiments are in progress in our laboratory. Polyadenylation may also play a role in processes other than translation and RNA stability. However, it is unlikely that polyadenylation regulates kRNA editing efficiency, since most never edited RNAs also contain both short and long poly(A) tracts (6).

The presence of kRNA editing is correlated with the presence of long poly(A) tracts since unedited RPS12 RNAs were found almost exclusively in subpopulations that contain short poly(A) tracts but partially edited and fully edited RPS12 RNAs were found in subpopulations containing both short and long poly(A) tracts. There are several mechanisms by which these two processes could be functionally connected. kRNA editing could create a *cis*-acting regulatory sequence in the RNA that is required for long poly(A) tract synthesis. Alternatively, the kRNA editing machinery (termed the editosome), which presumably assembles on the 3′-end of the RNA, could physically block the machinery that adds the long poly(A) tracts. As editing proceeds 3′→5′, the 3′-end of the RNA would be freed and the long poly(A) tract added. The editosome could also recruit the poly(A) polymerase and associated factors to the unedited RNA. All of these models suggest that the kRNA editing machinery and the polyadenylation machinery are physically associated or are able to process a given RNA concurrently.

The results presented here demonstrate that a portion of partially edited RPS12 RNAs contain long poly(A) tracts. Thus, in *T.brucei* mitochondria, the long poly(A) tract is probably not the lone signal for translation. The correlation between the presence of kRNA editing and the presence of a long poly(A) tract suggests that multiple RNA processing events in the mitochondria of *T.brucei* are coordinated. A thorough examination of the mechanisms by which these RNA processing events interact will increase our understanding of the unique methods of gene regulation in trypanosome mitochondria.

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