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Kinetoplastid RNA Editing: In Vitro Formation of Cytochrome b gRNA-mRNA Chimeras from Synthetic Substrate RNAs

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Summary

RNA editing in the kinetoplastid Trypanosoma brucei results in the addition and deletion of uridine residues within several mitochondrial mRNAs. The site and number of uridines added appears to be directed by small (~70 nt) guide RNAs (gRNAs), which can base pair to the edited sequences. We examined reactions involving synthetic cytochrome b (CYb) gRNA and pre-edited mRNA in vitro. A major product of the in vitro reaction is a chimeric RNA molecule containing both gRNA and mRNA sequences. Formation of the CYb gRNA-mRNA chimera was specific, since such molecules did not accumulate when either the gRNA or mRNA was substituted with control RNAs. The reaction required a free 3′ hydroxyl on the gRNA and was unaffected by capping of the gRNA's 5′ end. Direct RNA sequencing indicated that the CYb gRNA is covalently linked via its 3′ poly(U) tail to one of the editing sites on the CYb mRNA. These results suggest that the U's added during editing are donated by the poly(U) tail of a gRNA via a chimeric gRNA-mRNA intermediate.

Introduction

RNA editing in kinetoplastid mitochondria involves the addition and, to a lesser degree, deletion of uridine (U) residues at defined sites within the coding regions of several mRNAs (reviewed in Simpson and Shaw, 1989; Feagin, 1990; Benne, 1990). RNA editing is posttranscriptional, involving the modification of pre-edited precursor RNAs (Harris et al., 1990). The term "pre-edited" refers to a precursor mRNA that has not been modified by editing but will be edited in its mature form (terminology of Simpson and Shaw, 1989). The cytochrome b (CYb) mRNAs in all kinetoplastids examined thus far contain a single edited region near their 5′ end. In each case, editing generates an AUG that can be used as an initiation codon for the mRNA (Feagin et al., 1987, 1988).

The information required for formation of edited mRNAs from pre-edited precursors is found in small (~70 nt) "guide" RNAs (gRNAs) (Blum et al., 1990; Sturm and Simpson, 1990b; Pollard et al., 1990; van der Spek et al., 1991). This class of mitochondrial RNA can form continuous helices with edited sequences that can contain both GU and, to a lesser degree, AC pairing. gRNAs have sequences at their 5′ end that base pair to mRNA sequences 3′ of an editing site. This is followed by internal sequences, which are complementary to the edited version of an mRNA edited region, and a nonencoded 3′ poly(U) tail. Though gRNAs certainly exist in the steady-state RNA population, their precise role in editing remains unclear.

Numerous cDNAs from partially edited mRNAs have been analyzed (Abraham et al., 1988; Sturm and Simpson, 1990a; Decker and Sollner-Webb, 1990; Koslowsky et al., 1990). The mRNAs that these cDNAs represent are always edited in 3′ regions of the mRNA but not at 5′ editing sites, which has given rise to the notion that editing proceeds from 3′ to 5′ along the mRNA being modified. Many of these partially edited mRNAs have sequences at the boundary of 3′ edited and 5′ unmodified sequences that appear to have been incorrectly edited. This may mean that the editing activity adds the improper number of U's or adds U's to an improper site, but returns to correct these improprieties to form a correctly edited mRNA. Alternatively, it has been proposed that usage of incorrect gRNAs by the editing machinery gives rise to these incorrectly edited RNAs (Sturm and Simpson, 1990b).

Initially it was thought that the mechanism of editing involved endonuclease cleavage at an editing site, followed by addition of uridines to the newly generated 3′ hydroxyl of the 5′ cleavage product by a terminal uridylyltransferase (TUTase). Ligation of the broken RNA chain would be the last step in a single round of U addition. Detection of nonspecific TUTase and RNA ligase activities in the mitochondria of the kinetoplastid Leishmania tarentolae appeared to support this notion (Bakalara et al., 1989).

Recently, chimeric RNA molecules containing a gRNA covalently linked to a 3′ fragment of a partially edited mRNA have been detected by polymerase chain reaction (PCR) (Blum et al., 1991). The junctions between the mRNA and gRNA sequences are generally at editing sites. A model based on theoretical grounds (Cech, 1991) and on the structure of these chimeric RNA molecules (Blum et al., 1991) has been proposed in which editing occurs via multiple rounds of transesterification akin to RNA splicing reactions. First, the 3′ hydroxyl of a gRNA's poly(U) tail attacks the phosphodiester bond at an editing site, creating a chimeric gRNA–mRNA molecule and a 5′ fragment of the mRNA. In the second step, the 3′ hydroxyl of the 5′ mRNA fragment attacks a phosphodiester bond in the gRNA's poly(U) tail, generating a gRNA with a shorter U tail and an mRNA with extra U's added at the editing site. This model predicts that endoribonuclease and RNA ligase activities are not involved in editing. Also, U's are added not to the 5′ fragment of the mRNA by TUTase, but via the gRNA–mRNA intermediate. A mitochondrial activity that catalyzes this process has not yet been identified.

Our approach to understanding the mechanism of RNA editing has been to examine specific reactions that pre-edited precursor mRNAs undergo in vitro. In this study, we report in vitro formation of a chimeric RNA molecule containing synthetic gRNA sequences covalently linked to a fragment of a pre-edited mRNA. The reaction was specific for the CYb gRNA and mRNA substrates, since re-

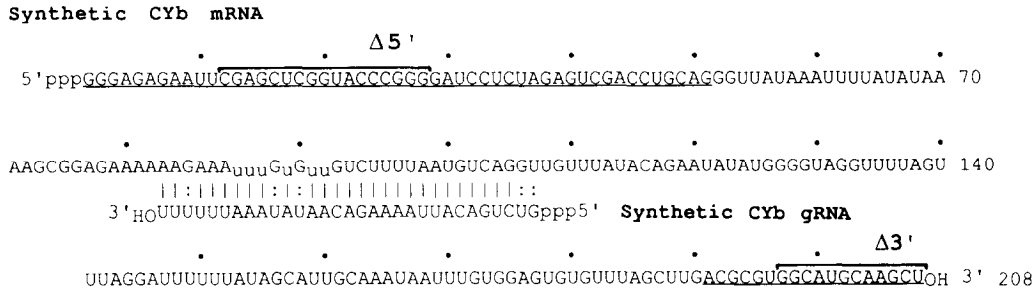

Figure 1. Sequence of Cytochrome b gRNA and mRNA Substrates

The synthetic CYb mRNA was transcribed from plasmid sequences with T3 RNA polymerase. It consists of 138 nt from the 5′ end of the T. brucei mitochondrial CYb gene, flanked by 51 nt and 18 nt of polylinker sequences (underlined) on the 5′ and 3′ ends, respectively. CYb gRNA was synthesized in vitro using T7 RNA polymerase and a synthetic DNA template (Milligan et al., 1987), based on the structure of CYb gRNA-I from Leishmania (Blum et al., 1991) and Crithidia (van der Spek et al., 1991). The sequence of the CYb gRNA is shown beneath the CYb mRNA sequence with which it interacts. The sites of U addition directed by this gRNA are shown in subscripted lowercase. The Watson–Crick base pairs that the CYb gRNA can form with the CYb mRNA are shown as solid lines between bases. GU base pairs are depicted by colons. For clarity, 32 additional editing sites, beginning 5 nt upstream from this editing site and directed by a second CYb gRNA in other kinetoplastids, are not shown explicitly. Lines above the CYb-pre mRNA sequence demarcate the sequences deleted in the CYb-preΔ5′ and CYb-preΔ3′ substrate RNAs as indicated.

placing either with control RNAs did not result in accumulation of chimeric RNA molecules. Blocking of the 5′ end of the gRNA by capping with GTP and guanylyl transferase did not effect chimera formation. Ligation of a 3′,5′ cytidine bisphosphate (pCp) moiety onto the 3′ end of the gRNA blocked chimera formation. Removal of the 3′ terminal phosphate of the pCp-labeled gRNA restored the ability to form the chimera. Direct RNA sequencing of the gRNA–mRNA chimera indicated that the synthetic gRNA was covalently linked by its 3′ poly(U) tail to one of the CYb mRNA editing sites. These results are consistent with an in vivo mechanism of kinetoplastid RNA editing where the added U's are donated by the 3′ poly(U) tail of a gRNA via a gRNA–mRNA chimeric intermediate.

Results

Incubation of Synthetic CYb gRNA and Pre-edited CYb mRNA with T. brucei Mitochondrial Extracts

The sequences of the synthetic CYb mRNA and gRNA are shown in Figure 1. The CYb pre-edited substrate RNA, CYb-pre, was transcribed in vitro from a cloned DNA template containing the 5′-most 138 nucleotides (nt) of the T. brucei CYb mRNA, including the entire region that will be edited in the mature mRNA. Flanking the CYb sequences in the RNA are 51 nt and 18 nt of polylinker sequences on the 5′ and 3′ ends, respectively. A gRNA for the 3′ portion of the CYb editing site was synthesized from a synthetic DNA template using T7 RNA polymerase (Milligan et al., 1987). Since gRNAs for the CYb editing site have not yet been reported in T. brucei, this gRNA was based on the sequence of the CYb-I gRNA from Leishmania (Blum et al., 1990) and is also similar in structure to the homologous gRNA in Crithidia fasciculata (van der Spek et al., 1991). This gRNA directs the addition of a total of six U's at the three 3′-most editing sites. A separate gRNA is apparently required for the editing of the remaining 32 editing sites, which begin 5 nucleotides upstream (Blum et al., 1990; van der Spek et al., 1991).

Figure 2 shows the result obtained when the CYb-pre RNA was incubated with a Triton X-100 extract from T. brucei mitochondria. 3′ end-labeled CYb-pre was cleaved by the extract in the vicinity of the 3′-most editing sites to yield a ~120 nt 3′ fragment (Figure 2, lane 2). When uniformly labeled CYb-pre was used in the in vitro reaction, the ~120 nt 3′ fragment and a corresponding, ~90 nt 5′ fragment were observed (Figure 2, lane 3). We next determined the effect of adding increasing amounts of synthetic CYb gRNA. In lanes 4–8, 1 pmol of uniformly labeled CYb-pre RNA and varying amounts of synthetic CYb gRNA, from 0.1 to 5 pmol, were incubated together with mitochondrial extract. As the amount of synthetic CYb gRNA was increased, a new reaction product of ~155 nt accumulated, while the amount of 3′ cleavage product decreased. A small amount of material running at ~155 nt was seen even in the absence of exogenous CYb gRNA.

The ~155 nt product may represent a new cleavage site induced by the addition of the synthetic CYb gRNA; however, the lack of a reciprocal cleavage fragment of ~55 nt makes this unlikely. A more likely possibility is that this product represents a chimeric RNA molecule, analogous to those observed in steady-state Leishmania mitochondrial RNA (Blum et al., 1991). Such a molecule would contain the synthetic CYb gRNA (~34 nt) and the pre-edited CYb mRNA 3′ cleavage fragment (~120 nt). Consistent with this idea is the decrease in the 3′ cleavage product as gRNA concentrations were increased.

Formation of the ~155 nt Product Is Specific for CYb mRNA and gRNA

We next determined if formation of the ~155 nt product was specific for the CYb substrate RNAs. In Figure 3A, two different control RNAs were substituted for the synthetic CYb gRNA in reactions with uniformly labeled CYb-pre RNA. Neither E. coli $tRNA_{phe}$ nor pGEM RNA, a 37 nt RNA generated by transcription of pGEM polylinker sequences, was able to stimulate formation of the ~155 nt product (compare lane 1 with lanes 2–5). Even when 5-fold

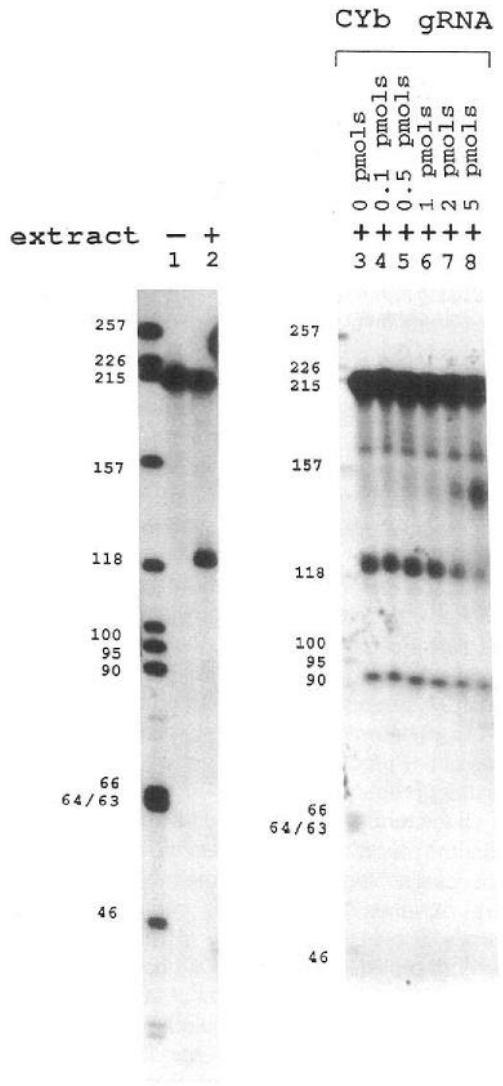

Figure 2. Incubation of CYb mRNA and gRNA Substrates with T. brucei Mitochondrial Extract

Radiolabeled CYb-pre RNA was incubated with mitochondrial extract in the presence or absence of synthetic CYb gRNA. In lanes 1 and 2, 3′ end–labeled CYb-pre was used, and in lanes 3–8, uniformly labeled CYb-pre was used. Either no gRNA was added (lanes 1–3), or varying amounts of CYb gRNA, from 0.1 to 5 pmol (lanes 4–8), was added as indicated. Reactions contained 1 pmol of radiolabeled CYb-pre RNA and 10 μl of mitochondrial extract in a 30 μl reaction. CYb-pre cleavage fragments migrate at ~120 nt for the 3′ fragment and ~90 nt for the 5′ fragment. The gRNA-specific product is seen at ~155 nt. Markers used throughout are an end-labeled AluI digest of pBluescript SK+ DNA.

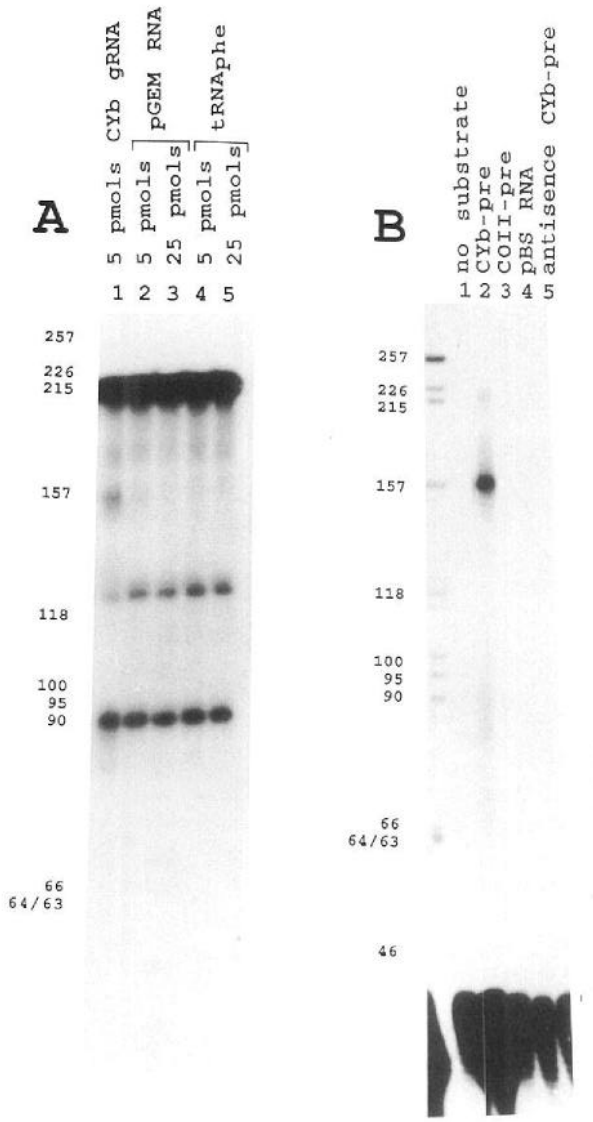

Figure 3. Specificity of the In Vitro Reaction

(A) Uniformly labeled CYb-pre RNA (1 pmol) was incubated with the indicated amounts of nonradiolabeled synthetic gRNA or control RNAs (E. coli $tRNA_{phe}$ and pGEM RNA, a 37 nt RNA transcribed from vector DNA sequences). In lane 1, 5 pmol of nonradiolabeled CYb gRNA was added. In lanes 2–5, the indicated amount of either pGEM RNA (lanes 2 and 3) or $tRNA_{phe}$ (lanes 4 and 5) was added.

(B) Incubation of various nonradiolabeled substrate RNAs with 1 pmol of CYb gRNA, uniformly labeled with [α^{32}P]UTP. In lane 1, the gRNA was incubated with extract alone. In lanes 2–5, 0.2 pmol of a nonradiolabeled RNA was added as indicated: lane 2, CYb-pre mRNA; lane 3, COII-pre, a 425 nt RNA containing the cytochrome oxidase subunit II editing site; lane 4, pBS RNA, a 360 nt RNA transcribed from pBluescript vector sequences; lane 5, the antisense version of CYb-pre mRNA.

more of the control RNAs were used (lanes 3 and 5), there was no substantial increase in the amount of this product. Additional control RNAs, including E. coli 5S rRNA, total cellular RNA, and various plasmid-derived RNAs, also failed to yield chimeric products (data not shown).

If the ~155 nt RNA is indeed a gRNA–mRNA chimera, then it should be possible to radiolabel the same product using labeled gRNA instead of labeled mRNA. In Figure 3B, synthetic CYb gRNA, uniformly labeled with [α^{32}P]-UTP, was incubated with various other RNAs in the presence of mitochondrial extract. When the labeled gRNA was incubated with extract alone, no additional RNA species were generated. When nonradiolabeled CYb-pre was added to the reaction, a prominent band at ~155 nt was observed. This product was not formed when the CYb-pre

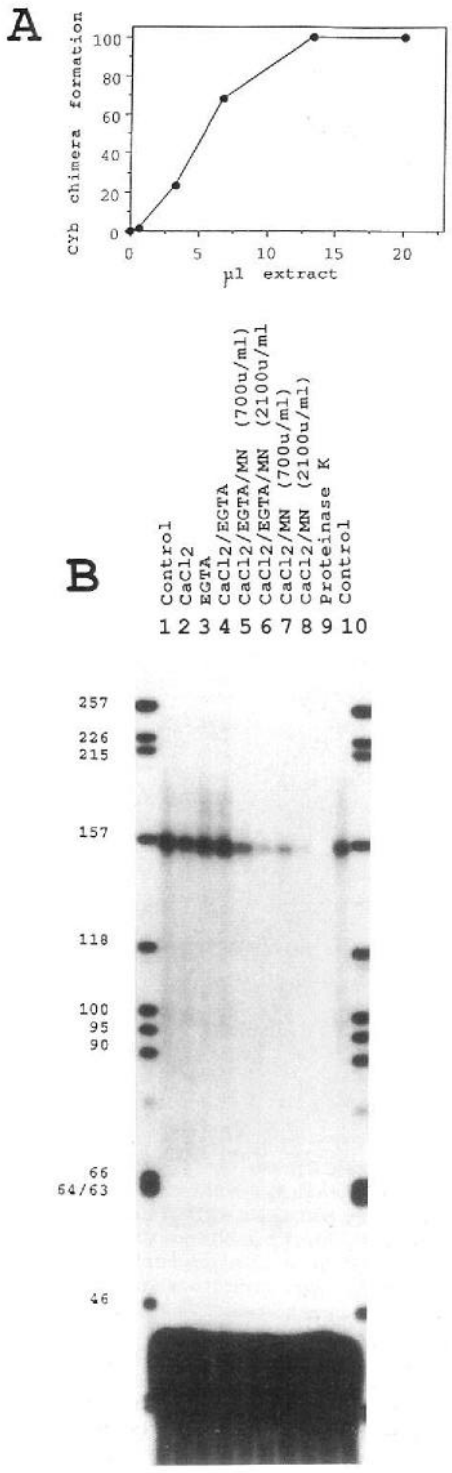

Figure 4. Characterization of the In Vitro Activity

(A) Extract dependence of the in vitro reaction. Reactions similar to that depicted in Figure 3B, lane 2, were performed using uniformly labeled CYb gRNA and nonradiolabeled CYb-pre mRNA. Reactions contained varying amounts of mitochondrial extract and reaction buffer to bring the total volume to 30 µl. The amount of gRNA incorporated into the ~155 nt gRNA-specific band was determined by densitometery and plotted as a percentage of the highest value observed.

(B). Effects of MN and proteinase K pretreatment on the in vitro reaction. Reactions contained 1 pmol of uniformly labeled CYb gRNA and 0.2 pmol of nonradiolabeled CYb-pre mRNA. Lanes 1 and 10 are control reactions where the extract used was preincubated for 30 min at 37°C. Lanes 2–4 are controls for preincubation with $CaCl_2$ and EGTA as indicated. In lanes 5–8, the extract used was either preincubated at 37°C for 30 min with the indicated amount of MN with EGTA (lanes 5 and 6) or EGTA was added after the preincubation (lanes 7 and 8). In lane 9, the extract used was preincubated with 50 µg/ml proteinase K for 30 min at 25°C.

substrate RNA was substituted with three different control RNAs—a 425 nt RNA containing the cytochrome oxidase subunit II editing site (COII-pre), a 360 nt RNA transcribed from pBluescript vector sequences (pBS RNA), and the antisense version of the CYb-pre RNA. Reactions were done in the presence of 1 mM nonradioactive UTP; therefore, it is unlikely that the ~155 nt RNA is labeled with uridine liberated via degradation of the input gRNA. Thus, a product of the same molecular weight (~155 nt) is observed using either labeled CYb-pre mRNA or labeled CYb gRNA. Formation of this product clearly requires both CYb mRNA and CYb gRNA.

Characterization of the In Vitro Activity

Figure 4A demonstrates the extract dependence of the formation of the putative gRNA–mRNA chimera. In this experiment, uniformly labeled CYb gRNA was incubated with nonradiolabeled CYb-pre mRNA in reactions similar to that in Figure 3B, lane 2. Here increasing quantities of mitochondrial extract were added and the amount of ~155 nt product was determined by densitometery. When the RNAs were incubated with buffer alone, no reaction products were detected. When increasing amounts of extract were added, a corresponding increase in the ~155 nt putative CYb gRNA–mRNA chimera was observed. Thus, formation of this product is dependent on factors present in the extract preparation.

To characterize the mitochondrial components necessary for the reaction, we pretreated the extract with either Micrococcal nuclease (MN) or proteinase K (Figure 4B). In Figure 4B, lanes 1 and 10 show control reactions with uniformly labeled synthetic CYb gRNA and nonradiolabeled CYb-pre RNA. Lanes 2–6 are control reactions for the MN pretreatment. In this type of analysis the extract is pretreated with MN, which requires calcium for activity, to destroy endogenous nucleic acids. Then EGTA is used to chelate the calcium, thereby inactivating the enzyme so that substrate RNAs can be added and activity assayed. Lanes 2–4 show that the activity is unaffected by preincubation with 1 mM $CaCl_2$ alone, 4 mM EGTA alone, or $CaCl_2$ and EGTA together. Since MN can also inhibit RNA processing reactions by binding to the substrate RNAs and not by digestion of extract components, control reactions with inactive MN (where the enzyme is added in the presence of EGTA) are also critical (Wang and Gegenheimer, 1990). In Figure 4B, lanes 5–8, the extract was pretreated with either 700 U/ml or 2100 U/ml MN for 30 min at 37°C, either in the presence of EGTA (lanes 5 and 6) or with EGTA added after pretreatment (lanes 7 and 8). Inhibition of the reaction by MN was indeed observed at these concentrations of enzyme, but was independent of whether the pretreatment was done with active or inactive enzyme.

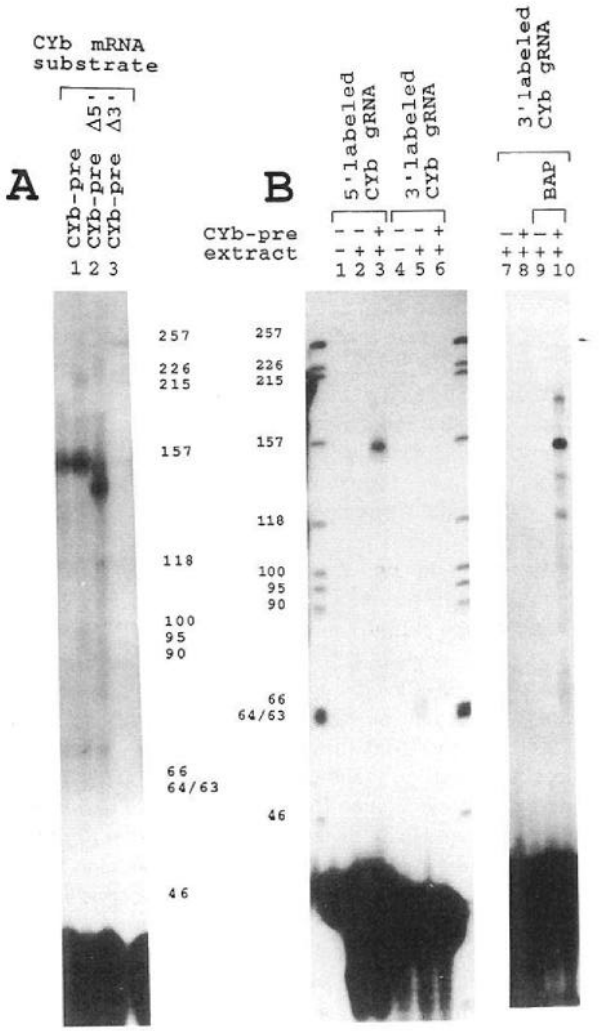

Figure 5. Characterization of the CYb gRNA–mRNA Chimera

(A) Effect of deletions of CYb-pre mRNA 5′ and 3′ polylinker sequences. Uniformly labeled synthetic CYb gRNA (1 pmol) was incubated with 0.2 pmol of CYb-pre mRNA (lane 1), CYb-preΔ5′ RNA (lane 2), or CYb-preΔ3′ RNA (lane 3). The sequences deleted in the CYb-preΔ5′ and CYb-preΔ3′ substrates are depicted in Figure 1.

(B) Effects of gRNA 5′ and 3′ modification on the chimera formation. Reactions contained 0.2 pmol of CYb-pre mRNA and extract as indicated. In lanes 1–3, 1 pmol of synthetic CYb gRNA that had been 5′-capped with [α^{32}P]GTP and guanylyl transferase was used. In lanes 4–8, the gRNA used was 3′-labeled with 3′,5′[5′-^{32}P]cytidine bisphosphate and T4 RNA ligase. In lanes 9 and 10, the same 3′ end–labeled gRNA used in lanes 7 and 8 was dephosphorylated with bacterial alkaline phosphatase prior to use.

Pretreatment of the extract with 200 U/ml MN did not inhibit the reaction to any detectable degree, while control reactions where EGTA was not added indicated that the MN was active under these conditions (data not shown). Careful comparison of the reactions done with active and inactive MN reveals that the extract pretreated with active MN showed a marginally greater degree of inhibition (1.5- to 2-fold; compare lane 5 with 7 and lane 6 with 8).

In contrast to the relative insensitivity to MN digestion, the activity was completely sensitive to pretreatment with 50 μg/ml proteinase K for 30 min at 25°C (Figure 4B, lane 9). The relative insensitivity to MN digestion is consistent with a lack of requirement for endogenous extract RNAs, although it may need nucleic acid components, which are protected from nuclease digestion. On the other hand, the sensitivity to pretreatment with proteinase K could mean that the activity is composed of or dependent on protein(s). It is unlikely that proteinase K inhibits the reaction by releasing inhibitory RNAs, since proteinase K–treated, phenol-extracted total mtRNA is not inhibitory (data not shown).

Characterization of the Putative CYb gRNA–mRNA Chimera

If the ~155 nt product is indeed a gRNA–mRNA chimera, analogous to those observed in steady-state mitochondrial RNA, then it would be composed of a single gRNA covalently linked, presumably 3′ to 5′, to a fragment of the mRNA that extends from an editing site to the 3′ end of the mRNA. Therefore, we determined the effect of deletions in the 5′ and 3′ polylinker sequences of CYb-pre on the migration of the putative gRNA–mRNA chimera (Figure 5A). CYb-preΔ5′ RNA has 17 nt of polylinker sequence deleted from its 5′ end, but is otherwise identical to CYb-pre RNA (see Figure 1). The putative CYb gRNA–mRNA chimera was unaffected by this deletion and migrated at ~155 nt when either CYb-pre or CYb-preΔ5′ was used (Figure 5A, lanes 1 and 2). CYb-preΔ3′ RNA has 13 nt of polylinker sequence deleted from its 3′ end (Figure 1). When this substrate RNA was used, the putative chimeric RNA decreased in size by the corresponding amount (lane 3). This shows that the ~155 nt products are indeed chimeras of some form, which contain both gRNA and mRNA sequences.

We next wished to examine whether the linkage to the CYb mRNA 3′ fragment involved the 5′ end or the 3′ end of the synthetic CYb gRNA. To block these termini, the synthetic gRNA was either capped on its 5′ end with [α^{32}P]GTP and guanylyl transferase or 3′ end–labeled using 3′,5′[5′-^{32}P]cytidine bisphosphate (pCp) and T4 RNA ligase. The 5′ capped CYb gRNA was converted to the gRNA–mRNA chimera in a reaction requiring both extract and CYb-pre substrate RNA (Figure 5, lanes 1–3). Since the reaction is not greatly affected by capping the 5′ end of the gRNA with GTP, it is likely that it is the 3′ end of the synthetic CYb gRNA that is involved in the linkage to the mRNA fragment. Consistent with this idea is the observation that the gRNA labeled on the 3′ end with pCp is unable to participate in chimera formation (lanes 4–6). The nature of the 65 nt product seen with pCp-labeled gRNA and extract alone (lane 5) is unknown.

Therefore, the reaction is blocked either by having a C at the 3′ terminus of the gRNA or by the presence of a phosphate on the 3′ hydroxyl of that C. Alternatively, the lack of chimera formation could be a concentration effect due to differences in specific activity. To distinguish between these possibilities, the pCp-labeled synthetic gRNA was dephosphorylated with bacterial alkaline phosphatase (BAP) (Figure 5B, lanes 9 and 10). Once again, synthetic CYb gRNA that was 3′ end–labeled with pCp was

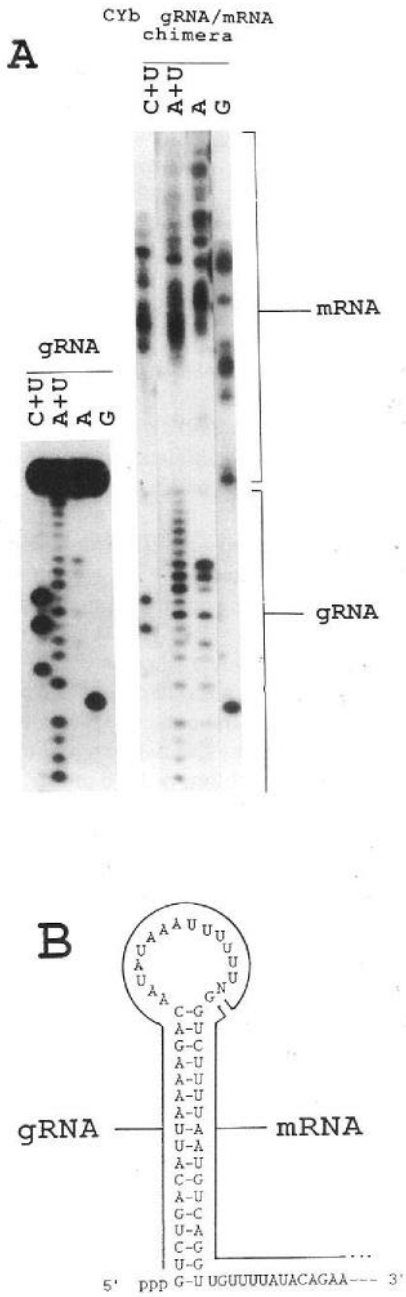

Figure 6. Sequence and Structure of the gRNA–mRNA Chimera

(A) Partial digestion of 5′ end–labeled CYb gRNA and gRNA–mRNA chimera with single-strand, base-specific endonucleases. The nucleotide specificity of the enzymes used is indicated above individual lanes. The origin of sequences in the gRNA–mRNA chimera is depicted by brackets at the right.

(B) Potential secondary structure of the junction region. Folding of the entire gRNA–mRNA sequence was modeled using the UWGCG program FOLD (Zuker and Stiegler, 1981; Devereux et al., 1984). The first 64 nucleotides of the predicted structure, which contain a 17 bp stem adjacent to the gRNA/mRNA junction point, are shown. As in (A), the gRNA and mRNA sequences are bracketed.

unable to form a chimera (lanes 7 and 8). In contrast, treatment of this preparation of gRNA with BAP restored the ability of the gRNA to form a chimera in the presence of CYb-pre and extract. In addition to the chimeric RNA, at least three additional products were also observed. These may represent side reactions that result from the presence of the remaining C on the 3′ end of the gRNA, or the presence of a hydroxyl on the 5′ end. The sensitivity of the reaction to the presence of a phosphate on the 3′ terminus of the gRNA indicates that it is this portion of the molecule that participates in the linkage to the mRNA sequences.

Examination of the gRNA–mRNA Junction

For a complete characterization of the CYb gRNA–mRNA chimera formed in vitro, we wished to examine the junction between the 3′ end of the synthetic CYb gRNA and the mRNA sequences. The CYb gRNA–mRNA chimera formed in vitro was refractory to the reverse transcription and PCR amplification strategy used to examine Leishmania chimeras in steady-state mitochondrial RNA (Blum et al., 1991), even though much greater quantities of target sequences and a variety of primers were used (data not shown).

As an alternative, direct sequencing of the chimera formed using 5′ end–labeled gRNA was attempted. Figure 6A shows the partial digestion of the CYb gRNA and chimera with single-strand, base-specific endoribonucleases. The 5′-labeled synthetic gRNA was easily sequenced with these enzymes at 55°C in 7 M urea. However, very little of the sequence of the chimera could be obtained using these conditions. Only when the chimera was preincubated for 10 min at 90°C in 7 M urea, then transfered immediately to 55°C, could the 5′ portion of the chimera be digested with these single-strand, base-specific enzymes. The difficulty in digestion of this portion of the chimera with single-strand, specific nucleases and our inability to amplify them by RT-PCR is likely due to the secondary structure of the chimeric RNA, which has the ability to form a continuous 17 bp helix (Figure 6B). Alternatively, some form of modification may make the chimeric RNA, in part, resistant to nuclease digestion.

The exact synthetic gRNA sequence can be read from the 5′ end of the chimera, including a 6 nt poly(U) tail (Figure 6). The next residue is read as an N, since digestion products are present in multiple lanes. This nucleotide most likely comes from the synthetic gRNA, which has some heterogeneity of sequence introduced at the very 3′ terminus by T7 RNA polymerase during synthesis in vitro (Milligan et al., 1987; Milligan and Uhlenbeck, 1989). Consistent with this idea is the observed distribution of nucleotides at the 3′ terminus of the in vitro synthesized gRNA. Nearest neighbor analysis of the synthetic gRNA, 3′ end–labeled with pCp, showed that 31.8% ended in U, 56.2% ended in A, 10.9% ended in C, and only 1.1% ended in G (data not shown).

Two G's from the mRNA are seen after this nucleotide, then no further digestion products are seen until the next G in the mRNA sequence. The spacing between these digestion products is 10 nt (± 1 nt). The adjacent G's at the junction could be G_{90} and G_{91} (see Figure 1), placing the junction at the middle editing site directed by the gRNA. Alternatively, the major G at the junction could be G_{91}, which would mean that the junction is at the 3′-most editing site, and the second, weaker G would be a consequence of

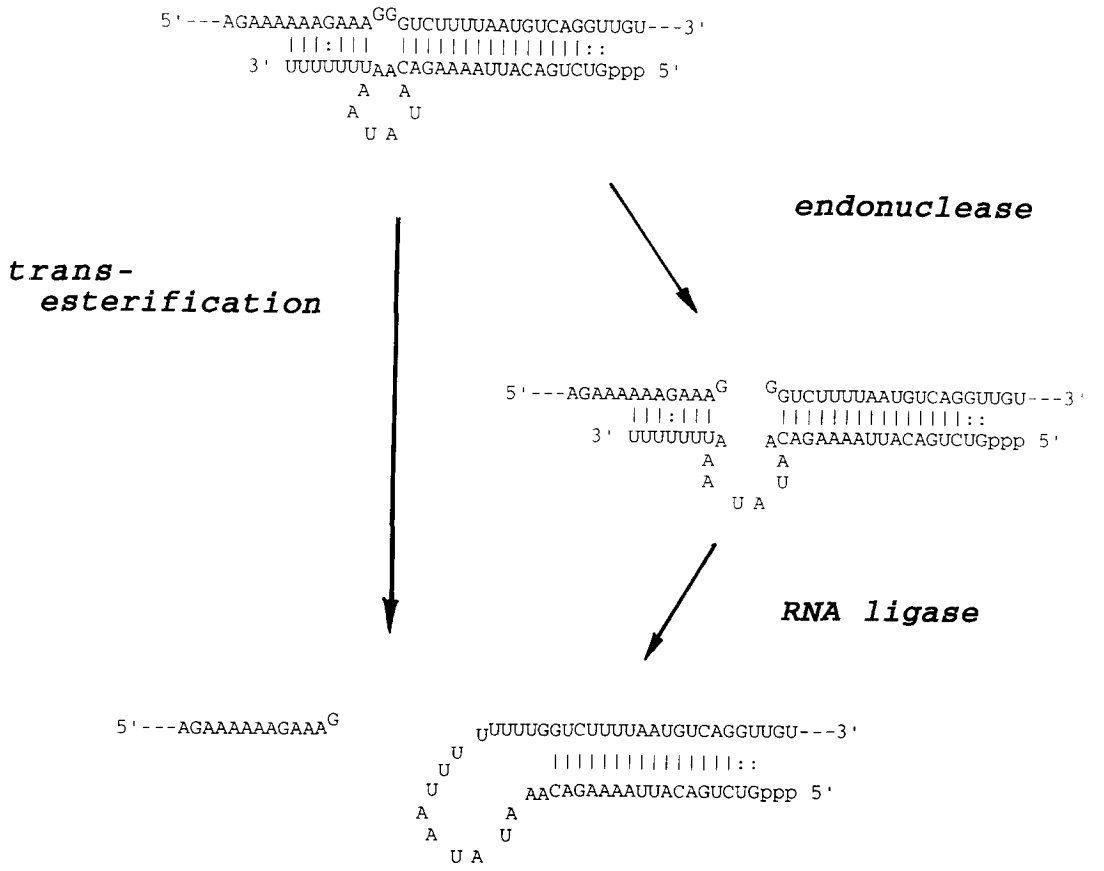

Figure 7. Two Potential Mechanisms for the Formation of gRNA–mRNA Chimeras

The proposed interaction of the synthetic CYb gRNA and CYb mRNA is shown at top. Watson–Crick base pairs are depicted by solid lines, while GU pairs are shown by colons. The proposed structure for the CYb gRNA–mRNA chimera is shown below. Theoretically, such a molecule could form in a single step by transesterification (Cech, 1991, Blum et al., 1991). A different mechanism is depicted to the right. A potential intermediate in the formation of the gRNA–mRNA chimera, center right, is one where the mRNA has been cleaved by an endonuclease activity. Subsequent formation of the chimera would involve an RNA ligase activity.

the heterogeneity of the synthetic gRNA. Thus, the gRNA could be linked to either of these two adjacent editing sites. Although the exact junction site could not be determined without ambiguity, we have shown that it consists of the 3′ poly(U) tail of the synthetic gRNA, covalently linked to one of the editing sites on the CYb-pre mRNA.

Discussion

The following observations demonstrate the formation of CYb gRNA–mRNA chimeras in vitro, analogous to those observed in steady-state kinetoplastid mitochondrial RNA: Molecules migrating at ~155 nt, the predicted size for a gRNA–mRNA chimera containing synthetic CYb gRNA (~34 nt) and CYb-pre mRNA sequences 3′ of the 3′-most U addition site (~120 nt), accumulate when CYb-pre mRNA and CYb gRNA are incubated in the presence of extract. These products can be visualized using either labeled CYb-pre mRNA or labeled CYb gRNA. These products are sensitive to deletions in the 3′ portion of CYb-pre mRNA but are unaffected by deletions 5′ of the editing site. The reaction was sensitive to the presence of a phosphate on the 3′ end of the synthetic gRNA. Direct sequencing of these RNAs showed the gRNA linked via its poly(U) tail to one of the editing sites on the CYb-pre mRNA.

Of great interest is the mechanism by which the chimeric RNAs are formed. The transesterification model (Figure 7) proposed by Cech and Blum et al. suggests that chimera formation is a one-step process involving phosphodiester bond exchange. No endonuclease or RNA ligase activities are involved in this model, and a free 3′ mRNA cleavage product is not an intermediate of chimera formation. The fact that we observe chimera formation in vitro appears to support such a mechanism. This model would argue that the endonuclease cleavage we observe in the absence of exogenous gRNA (Figure 2, lanes 2 and 3) is not involved in the editing process. It may be that this reaction is similar to splice site hydrolysis observed with catalytic RNAs (Cech, 1990).

An RNA ligase activity has been detected in mitochondrial extracts from Leishmania (Bakalara et al., 1989), and a similar activity has also been detected in T. brucei mitochondrial extracts (V. W. Pollard, M. E. H., and S. L. H., unpublished data). Thus, it is possible that chimera forma-

tion can occur, not by a single transesterification step, but by a two-step mechanism involving endonuclease cleavage and ligation (Figure 7). The cleavage–ligation model predicts that the 3′ cleavage product generated in the absence of exogenous gRNA is an intermediate in the formation of the gRNA–mRNA chimera.

Though a parallel between RNA editing and group I intron splicing can be drawn (Cech, 1991; Blum et al., 1991), it does not appear to extend to self-catalysis. The in vitro reactions that we observe clearly require mitochondrial extract. The fact that chimera formation is sensitive to proteinase K suggests that protein components are necessary. In addition, the activity was not obviously sensitive to MN. However, there was a small degree of inhibition in the presence of active nuclease. The extract could contain nucleic acids that are protected from nuclease digestion. Further purification and characterization will be necessary to fully resolve the chemical nature of the chimera-forming activity.

Another area of interest is the precise structure of the gRNA–mRNA chimeras. The linkage between the synthetic gRNA and mRNA in the chimera formed in vitro appears to be identical to that found in vivo. Since the in vivo chimeras were detected by reverse transcription followed by PCR, they presumably contain a 3′-5′ phosphodiester bond between the gRNA and mRNA sequences. The experiments presented in Figures 5 and 6 are consistent with this idea. Blocking the 5′ end of the gRNA with GTP and guanylyl transferase did not affect chimera formation as might be expected. Blocking the 3′ end with pCp and RNA ligase destroyed the ability of the gRNA to participate in chimera formation. Removal of the 3′ phosphate with BAP restored chimera formation, indicating that a free 3′ hydroxyl on the gRNA is required for chimera formation. Alternatively, a phosphate on the gRNA 3′-OH could stearically block chimera formation via the 2′-OH. The sequencing analysis, though incomplete, is also consistent with the structural similarity of the chimera formed in vitro to those detected in vivo. The ability of 3′ pCp-labeled, BAP-treated gRNA to form chimeras indicates that a 3′ terminal U on the gRNA is not an absolute requirement for chimera formation. Consistent with this is the heterogeneity at the gRNA–mRNA junction region as determined by direct RNA sequencing.

The fact that gRNA–mRNA chimeras are also observed in vivo indicates that the in vitro reaction has some relevance for the mechanism of RNA editing. Now that a portion of the editing reaction is accessible to in vitro manipulation, it should be possible to examine further steps in the editing reaction, as well as the substrate and enzymatic requirements for chimera formation.

Experimental Procedures

Synthesis and Labeling of RNAs

pCYb-pre was a generous gift from B. Sollner-Webb,and its construction is described in Decker and Sollner-Webb (1990). pCYb-preΔ5′ was created by digestion of pCYb-pre with EcoRI and BamHI, which removed 17 bp of polylinker sequence from between the T3 promoter of pCYb-pre and the insert sequences. The digested DNA was incubated in the presence of the Klenow fragment of DNA polymerase I (BRL) and deoxynucleoside triphosphates to form blunt ends. The plasmid was resealed with T4 DNA ligase (BRL).

For synthesis of CYb-pre and CYb-preΔ5′, the appropriate plasmids were digested with HindIII, and T3 RNA polymerase (BRL) was used according to the manufacturer's recommendations. CYb-preΔ3′ RNA was synthesized from pCYb-pre digested with MluI, and pBS RNA was synthesized from pBluescript SK+ digested with PvuII. T3 polymerase was used for both RNAs. pGEM RNA, a 35 nt RNA generated from pGEM vector sequences, was synthesized by digesting pGEM DNA with XbaI and transcribing with SP6 polymerase (BRL). Uniformly labeled substrate RNAs were synthesized by performing the riboprobe synthesis reaction in the presence of 50 μCi of [α^{32}P]CTP or [α^{32}P]UTP (400Ci/mmol) and 500 μM each of the other nucleoside triphosphates in a 20 μl reaction. The apparent discrepancy in abundance of 5′ and 3′ cleavage products in Figure 2 versus Figure 3A is due to the differential distribution of labeled nucleotides in the uniformly labeled substrates. In Figure 2 the CYb-pre substrate was uniformly labeled with [α^{32}P]-UTP, while that used in Figure 3A was labeled with [α^{32}P]CTP.

CYb gRNA was synthesized in vitro by the method of Milligan et al. (1987), using T7 RNA polymerase and a synthetic DNA template composed of the following two oligonucleotides:

5′-TAATACGAGTCACTATA-3′
3′- ATTATGCTGAGTGATATCAGACTGTAATTTTCTGTTATATTTAA-AAAA-5′

The RNA synthesized from this template included the full-length RNA as well as equal amounts of RNAs from 1–5 nt longer, which were likely the result of stuttering of the polymerase at the terminal A residues of the template (Milligan et al., 1987; Milligan and Uhlenbeck, 1989). Cold gRNA (200 μl) synthesis reactions contained both oligos at a concentration of 500 nM; ATP, GTP, CTP, and CTP at 1 mM each; 40 mM Tris–HCl (pH 8.0), 20 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM spermidine, 0.001% Triton X-100, 50 mg/ml bovine serum albumin, and 200 U of T7 RNA polymerase. Uniformly labeled CYb gRNA was made in the same fashion, but cold UTP was substituted with 250 mCi of [α^{32}P]UTP at 400 Ci/mmol. RNAs were 5′ end–labeled with [α^{32}P]GTP and guanylyl transferase (BRL) and 3′ end–labeled with pCp and RNA ligase (BRL) as described (Pollard and Hajduk, 1991). To dephosphorylate pCp-labeled CYb gRNA, 20 pmol of labeled RNA was treated with 50 U of bacterial alkaline phosphatase (BRL) in 10 mM Tris (pH 8.0), 1 mM EDTA for 90 min at 37°C in a 20 μl reaction.

Substrate RNAs were gel-purified prior to use on 15% polyacrylamide–8 M urea gels for the synthetic gRNA and pGEM RNA, or 4% polyacrylamide–8 M urea gels for all other RNAs. Radiolabeled RNAs were visualized by autoradiography and nonradioactive RNAs by UV shadowing. The appropriate regions of the gel were excised and eluted overnight in 0.5 M ammonium acetate, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% sodium dodecyl sulfate. The eluted RNA was extracted with equal volumes of phenol–chloroform (1:1) and with chloroform, and then recovered by ethanol precipitation.

Preparation of T. brucei Mitochondrial Extracts and In Vitro Reactions

Mitochondria from exponentially growing procyclic trypanosomes were prepared essentially as described (Harris et al., 1990). Mitochondria from 1 liter of procyclic culture (2×10^{10} cells), containing about 12 mg of mitochondrial protein, were resuspended in 1 ml of 25 mM Tris–HCl (pH 7.9), 10 mM magnesium acetate, 1 mM EDTA, 60 mM KCl, 0.5 mM DTT, 10% glycerol, 1 mM ATP. Triton X-100 (10%) in sterile H_2O was then added to a final concentration of 0.5%. After 2 min of gentle mixing at room temperature, the solubilized mitochondria were placed back at 4°C and centrifuged at 10,000 × g in a microcentrifuge for 5 min, and the supernatant was assayed for activity. Aliquots of this mitochondrial extract were stored at −70°C for up to 2 months without detectable loss in activity.

In vitro assays typically contained CYb-pre mRNA and CYb gRNA in a ratio of 1:5. Exact concentrations and exceptions are detailed in the figure legends. These RNAs were mixed in 20 μl of 25 mM Tris/HCl, 10 mM magnesium acetate, 1 mM EDTA, 60 mM KCl, 0.5 mM DTT, 10% glycerol, 1 mM ATP. Then 10 μl of extract was added and the reaction incubated at 25°C for 90 min, at which time 200 μl of 40

mM Tris–HCl (pH 8.0), 50 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate, 50 μg/ml Proteinase K (BRL) was added to stop the reaction. This mixture was further incubated for 15 min at 25°C. Samples were extracted once with phenol–chloroform (1:1) and once with chloroform. One microgram of yeast tRNA, 20 μl of 3 M sodium acetate, and 570 μl of ethanol were added to precipitate the reaction products. Precipitated RNAs were recovered by centrifugation, washed once with 70% ethanol, and run on 6%, 7%, or 8% polyacrylamide–8 M urea gels. Radiolabeled RNAs were visualized by autoradiography.

For MN (Pharmacia) and proteinase K (BRL) pretreatments, 10 μl of extract was incubated with MN at 700 U/ml or 2100 U/ml for 30 min at 37°C or with 50 μg/ml proteinase K for 30 min at 25°C. EGTA and $CaCl_2$, when included in the reaction, were at concentrations of 4 mM and 1 mM, respectively.

RNA Sequencing

For sequencing of the chimera, a reaction using 5′-labeled gRNA and nonradiolabeled mRNA was scaled up 10-fold but otherwise processed normally. The chimeric RNAs were resolved on a 4% acrylamine–8 M urea gel and excised as described above. The chimera and unreacted 5′-labeled gRNA were sequenced using base-specific endoribonucleases: T1 (G-specific), U2 (A-specific), PhyM (A+U), and B. cereus (C+U) (Pharmacia) in 7 M urea using the buffers supplied by the manufacturer. For the chimeric RNA, reactions were heated to 90°C for 10 min and then transfered immediately to 55°C. While the reactions were kept at 55°C, enzyme was added and the incubation continued for 15 min. For CYb gRNA, enzyme was added to the reactions on ice and then incubated for 10 min at 55°C. Reactions were stopped by bringing them to −70°C in a dry ice–ethanol bath. A 15% polyacrylamide–8 M urea gel was used to resolve the partial digests.

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