Activation of Guide RNA-directed Editing of a Cytochrome b mRNA*

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Received for publication, April 10, 2000, and in revised form, July 31, 2000. Published, JBC Papers in Press, August 11, 2000, DOI 10.1074/jbc.M03002200

The coding sequence of several mitochondrial mRNAs of the kinetoplastid protozoa is created only after the addition or deletion of specific uridines. Although in vitro systems have been valuable in characterizing the editing mechanism, only a limited number of mRNAs are accurately edited in vitro. We demonstrate here that in vitro editing of cytochrome b mRNA is inhibited by an A-U sequence present on both the 5′-untranslated sequence and on a cytochrome b guide RNA. Mutation of the sequence on the guide RNA stimulates directed editing and results in the loss of binding to at least one component within the editing extract. Mutation of the sequence on the mRNA increases the accuracy of the editing. Evidence is provided that suggests the A-U sequence interacts with the editing machinery both in vitro and in vivo.

In vitro studies suggest that editing of several mitochondrial mRNAs of the kinetoplastid protozoa occurs through a cleavage/ligation mechanism (1–3). The binding of a guide RNA (gRNA)1 to the pre-edited mRNA immediately 3′ of the editing sites creates a recognition site for an endonuclease. U-addition by a terminal uridylyltransferase activity or U-deletions by an exonuclease activity occur at the 3′-OH created by the endonuclease. The number of U-insertions and deletions is determined by a guiding sequence on the gRNA. Re-ligation of the 5′ and 3′ fragments completes one editing cycle. The mRNA is progressively edited in an overall 3′ to 5′ direction through reiteration of the process at subsequent sites (reviewed in Ref. 4).

Although in vitro systems have been critical in defining the editing mechanism, they catalyze a complete editing cycle with only a limited number of mRNA substrates. Fractionated mitochondrial extracts prepared from Trypanosoma brucei catalyze both U-deletions from the first editing site and U-insertions into the second editing site of ATPase synthetase subunit 6 mRNA (2, 3). The same system also supports editing of a NADH dehydrogenase 7 (ND7)-ATPase 6 chimeric mRNA (3). However, a complete editing cycle of cytochrome b mRNA has not yet been demonstrated with these extracts, even though products are formed in the reaction that are consistent with the expected editing intermediates (5). Leishmania tarentolae mitochondrial extracts support gRNA-directed U-insertions into the first editing site of ND7 mRNA, but the guiding of insertions into a cytochrome b transcript could likewise not be demonstrated with this system (6). This is true, even though the cytochrome b mRNA isolated from the L. tarentolae mitochondria used to prepare the editing extracts is mostly edited (7).

Cytochrome b mRNA is one of two known L. tarentolae pre-edited mRNAs that undergo in vitro U-additions independent of gRNA (8). These insertions do not create a functional coding sequence, and their relevance to the gRNA-directed reaction remains unclear. Intermediates produced during the gRNA-independent reaction, however, are consistent with the cleavage/U-insertion/ligation mechanism proposed for the directed reaction (9). A 34-nucleotide untranslated A-U sequence element located immediately upstream of the cytochrome b editing sites was found to be necessary for the gRNA-independent U-insertions, and the majority of these U-insertions occur immediately 5′ and 3′ of the A-U sequence. The A-U element itself is sufficient to induce U-insertions when placed within an unedited mammalian transcript. This element was found to match a sequence within cytochrome b gRNA I at 20 out of 22 nucleotides (Fig. 1). The similarity in the two sequences suggested that it could also be involved in gRNA-directed editing, perhaps acting as a binding site for editing components (9). However, the lack of an in vitro system supporting gRNA-directed editing of cytochrome b made it difficult to assess its significance.

In this paper we describe an in vitro system that catalyzes gRNA-directed insertions into a cytochrome b mRNA transcript; accurate editing was confirmed by sequencing. Mutation of the A-U sequence on the gRNA stimulates gRNA-directed in vitro editing of the cytochrome b mRNA, whereas deletion of the corresponding mRNA A-U sequence significantly increases the fidelity of the guided reaction. In addition, mutations to the A-U sequence result in the loss of binding to at least one component of the editing extract, and results are suggestive that the A-U sequence also interacts with the editing machinery in vivo.

EXPERIMENTAL PROCEDURES

Mitochondrial Extract Preparation—A 500-ml culture of L. tarentolae (UC strain) was grown to an approximate density of 5 × 10^8 cells/ml in BHI medium (Difco) supplemented with 10 μg/ml hemin. The culture was then diluted 3-fold in fresh media and grown to a density of 1.6 ± 0.2 × 10^9 cells/ml, at which point the cells were harvested and mitochondria prepared as described previously (9). The mitochondria were resuspended in 950 μl of solubilization buffer (25 mM Hapes, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 20 mM KCl, 0.1 mM ATP, 1 mg/ml Pefabloc, 10 μg/ml leupeptin, and either 10% glycerol or 10 mg/ml BSA) for every 500 ml of starting culture. The resuspended mitochondria were solubilized with 0.5% Triton X-100 and stored frozen at −80 °C. Extracts stored in BSA were used without further fractionation in the editing assays. The protein concentration of the extract, prior to the addition of BSA, was approximately 2 μg/μl; protein concentrations were determined using a bicinchoninic acid-based method (Pierce).

The glycerol-containing extract was thawed on ice, and either it was loaded onto a 10–40% sucrose gradient as described (9) or a 500-μl aliquot was fractionated on a 1-ml Q Sepharose column (Amersham)
Pharmacia Biotech). The Q Sepharose column was equilibrated at 4°C with Buffer A (25 mM Hepes, pH 7.5, 10 mM MgCl₂, 0.5 mM dithiothreitol, 10% glycerol, 1 mg/ml Pefabloc SC, and 10 μg/ml leupeptin) containing 100 mM KCl. After loading the mitochondrial extract, the column was washed for four column volumes with Buffer A containing 100 mM KCl. The column was then washed until the conductivity of the column buffer returned to the 100–300 mM KCl gradient in Buffer A. The column flow rate was 0.2 ml/min, and 500–μl fractions were collected. The protein concentration of the fractions was determined using a Coomassie protein assay reagent (Pierce) and the KCl concentration determined with a conductivity meter.

With the exception of fractions used for gel shift assays, BSA was added to final concentration of 1 mg/ml to each of the collected 0.5 ml sucrose gradient or Q Sepharose fractions, and the fractions were concentrated 2–5-fold using Microcon 10,000 molecular weight cut-off microconcentrators (Millipore). The concentrated Q Sepharose fractions were diluted with Buffer A and re-concentrated to reduce the KCl concentration.

**RNA Transcripts**—T7 RNA polymerase was used to directly transcribe the cytochrome b gRNAs from the oligodeoxynucleotides listed below (10). The cytochrome b mRNA transcripts were transcribed from PCR products amplified from plasmid pM1. This plasmid, which encodes the four point mutations within the gRNA binding site (Fig. 1B), was constructed using a PCR overlap extension technique (11). A cloned maxicircle fragment encoding cytochrome b mRNA was used as a template. The overlapping mutagenic primers were forward primer 1115004 and reverse primer 1115005, and the two external primers were 8494 and 9473. The resulting PCR product was digested with EcoRI and cloned into Bluescript II KS (+) (Stratagene) and confirmed by sequencing. The PCR templates for the mRNA transcripts were then amplified from pM1 using either forward primer 5692 (wild-type A-U sequence) or 10210 (A-U deletion). The reverse primer 5691 was used for both reactions. This primer results in a 27-nucleotide tag at the 3' end of these transcripts, facilitating their discrimination from endogenous cytochrome b mRNA. The transcript containing the mRNA A-U sequence used for both the gel-shift assay and the attempt to stimulate gRNA-independent U-insertions in trans was transcribed directly from oligodeoxynucleotide 10899.

**Editing Assays**—The gRNA-directed reaction conditions are a modification of a previous procedure (6). A mRNA transcript (1 pmol) and 5 pmol of a gRNA in 25 mM Hepes, pH 7.9, and 0.2 mM EDTA, pH 8.0, were heated in a 10 μl volume at 65 °C for 3 min. The RNAs were added to 25 μl of editing buffer (100 mM Hepes, pH 7.9, 40 mM KCl, 20 mM MgCl₂, 6 mM potassium phosphate, pH 7.6, 2 mM dithiothreitol, 2 mM ATP, 2 mM GTP, 2 mM UTP, 1 mg/ml Pefabloc SC, and 10 μg/ml leupeptin) and incubated at 37°C for 10 min before incubation at 27°C for 1 h. The addition of EDTA, pH 8.0, to 0.2 mM, SDS to 0.5%, and proteinase K to 200 μg/ml terminated the reactions. After an additional 30-min incubation at 37°C, the reactions were phenol/chloroform-extracted and ethanol-precipitated.

The gRNA-directed U-insertions were detected with a primer extension assay synthesized from the extract-treated transcripts using primer 5693, which is complementary to the 3' tag sequence. The cDNA was ethanol-precipitated and half of it amplified by single-stranded PCR using either 32P-end-labeled 5692 or 32P-end-labeled 10210. Within any given set of reactions, the same primer was always used. These primers are sense to sequences upstream of the editing site, while the other clones were sequenced by T-tracking or 4-base sequencing of selected regions. The region on the L. tarentolae maxicircle transcript containing the gRNA sequence was amplified from mitochondrial DNA with a forward primer (15777) complementary to the upstream gene, cytochrome oxidase III, and reverse primer 7276, which is complementary to sequence downstream of the cytochrome b editing sites. The PCR product was cloned into Bluescript KS (+) for sequencing.

**Oligodeoxynucleotides**—The oligodeoxynucleotides shown below were used in this study.

**Templates for the Transcription of gRNAs**—The mutation to the A-U element and the number of guiding nucleotides for the first editing site are indicated in parentheses: 15042 (wild type; 3), TATTATTATTTATTACCTTAACATGCTATAGTGTAGCTGTTATTA; 15161 (Block 1 +3; 3), TATTAGGAAAAAAAGGGATACGATCGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 17169 (Block 1 +5; 3), TATTATTATTTATACACGATCTTTATTTGATACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 17168 (Block 3; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 17168 (Block 3; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA; 720183 (Block 2; 3), TATTATTATTTAAAGGATATATTATTATACACGATCTTTTACCTTTAACTTACATGCTATAGTGTAGCTGTTATTA.
RESULTS

gRNA-directed Editing of a Cytochrome b Transcript—A primer extension assay was used to detect in vitro gRNA-directed U-insertions occurring within the first editing site of a pre-edited cytochrome b transcript (Fig. 2A). This 205-nucleotide transcript contains all 15 cytochrome b mRNA editing sites and has four point mutations within the gRNA binding site (Fig. 1B). These mutations inhibit endogenous cytochrome b gRNA from interacting with the transcript and also permit it to be distinguished from the endogenous cytochrome b mRNA. Compensatory changes were made to the cytochrome gRNAs so as to maintain base pairing with the mutated gRNA binding site of the mRNA transcript. In addition, the number of U nucleotides guiding U-insertions into the first editing site was annealed to a template derived from the extract preparations, yet efforts at further optimizing the mitochondrial extract under the assay conditions failed to improve the signal enough to allow characterization of the reaction. The same extracts, however, consistently supported accurate editing of a ND7 mRNA at quantifiable levels (data not shown, and Ref. 6), suggesting that the editing system itself was not fundamentally flawed.

The cytochrome b gRNA I contains an A-U rich region with a 20 out of 22 nucleotide match to a sequence within the 5'-untranslated region of the cytochrome b mRNA (Fig. 1A). Since we had previously demonstrated that the mRNA A-U sequence stimulates the gRNA-independent reaction (9), the effect of the gRNA-A-U sequence on the gRNA-dependent reaction was investigated. Three blocks of U nucleotides within the aligned region of the cytochrome b gRNA I were mutated to C nucleotides (Fig. 1A). These A nucleotides normally function as guiding nucleotides for the 3rd, 5th, and 6th editing sites and as such do not base-pair with the pre-edited mRNA transcript. Mutation of any one of the A blocks on the gRNA increased U-insertion activity (Fig. 2B, lanes 5–10). The predominant +3 product is consistent with correct editing, but a ladder of extension products also occurs that is presumably derived from subpopulations of transcripts having different numbers of inserted U nucleotides. Incubation of radiolabeled mutated gRNAs with the mitochondrial extract under the assay conditions indicated that the mutations did not alter the stability of the RNA (data not shown). Likewise, native gel analysis indicated that differences in the annealing efficiency of the mutated gRNAs to the mRNA cannot solely account for the differences in the gRNA-directed reaction (data not shown).

Mutation of all three A blocks together (Fig. 2B, lane 10) or a double mutation of blocks 1 and 3 (Fig. 1B, lane 8) had the greatest effect on editing. Assigning the triple block mutation as 100% activation of gRNA-dependent editing, the effect of single block mutations ranged from 21 ± 1% (n = 2) activation for the block 1 mutation alone to 50 ± 10% (n = 3) activation for the block 3 gRNA mutation (Fig. 2B, lanes 4–6). The editing
activity of the block 1+2 double mutant was also 50 \pm 10\% (n = 3), while mutation of blocks 2+3 had 60 \pm 10\% (n = 3) of the activity of the triple mutant. Thus, whereas mutation of the A-U sequence on the mRNA inhibits the gRNA-independent reaction, mutation of the same sequence on the gRNA activates the gRNA-dependent reaction.

In order to determine whether the U-insertions are gRNA-directed, several gRNAs containing the triple block mutation and a different number of guiding nucleotides for the first editing site were used in the editing reaction (Fig. 2C). Each band of increasing size within the primer extension ladder contains increasing numbers of incorporated radiolabeled dA, and as a result the absolute intensity of each needs to be normalized for this increase in specific activity. However, even without the normalization, it is clear that changing the number of guiding nucleotides results in an increased intensity of the primer extension band consistent with correct editing. As the number of guiding nucleotides is increased from 2 to 5, there is a clear decrease in the corresponding editing signal. In addition, the band immediately above the signal consistent with correctly edited molecules is diminished relative to the rest of the ladder, as is especially evident for the gRNAs directing 4 and 5 U-insertions. The reason for these effects is not completely clear but could be related to the structure of the gRNA duplex being impacted as the number of guiding nucleotides is increased.

To confirm that the cytochrome b transcript is correctly edited in a gRNA-directed manner, individual RNAs with U-insertions were cloned and sequenced. The in vitro gRNA-directed reaction occurs at a low level (<1% of the RNA population) making direct cloning and sequencing of the edited subpopulation a challenge. We previously demonstrated that 4-thiouridine can be incorporated by the mitochondrial extract into the cytochrome b mRNA (9). Mercaptide formation between the 4-thiouridine and an organomercurial matrix facilitated the partitioning of the RNAs containing U-insertions. To clone and sequence individual molecules, the enriched RNA was reverse-transcribed and used as template for PCR. For this set of reactions, an oligodeoxynucleotide annealing just upstream of the first editing site and containing a single T at its 3’ end was used as the forward primer. Use of this primer further favored the amplification of those cDNAs derived from edited transcripts, as the primer would be mismatched when annealed to an unedited sequence. However, even with this selection the discrimination is not absolute, as unedited molecules are still amplified, although to a lesser extent (data not shown). The PCR products were cloned and sequenced.

Of the 40 clones sequenced, 11 had 3 T-insertions at editing site 1, which is consistent with the number of insertions expected from the +3 gRNA used in the editing reaction. A sequence representative of these clones is shown in Fig. 2D. This is the first demonstration of in vitro gRNA-directed editing of a cytochrome b mRNA. The remaining clones had 1 T insertion. These clones could have resulted either from the insertion of 1 U during the editing reaction or alternatively, from the extension of the mismatched forward primer when annealed to an unedited molecule. No clones were sequenced with 2 insertions, nor were any found with more than 3 as would have been expected from the primer extension ladder (Fig. 2B, lane 10). However, this bias could have resulted from thioUTP not being as good a substrate for the editing reaction as UTP and RNAs with fewer numbers of thiouridine insertions not binding as well to the organomercury matrix.2

The mRNA A-U Element Affects the gRNA-directed Reac-

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2 G. Connell, unpublished results.
Role of an A-U Sequence during Editing

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As deletion or substitution of the mRNA A-U element inhibits the gRNA-independent reaction and mutation of the A-U element of the gRNA activates guiding, the significance of this mRNA sequence to the gRNA-directed reaction was also investigated. Deletion of the mRNA A-U element did not abolish the gRNA-directed U-insertions (Fig. 3A). Rather, this deletion increased the accuracy of the gRNA-directed reaction relative to mRNA transcripts containing the A-U sequence by a factor of 1.7 ± 0.3 (n = 3; Fig. 3B). The increased fidelity suggests that the presence of the mRNA A-U sequence in some manner constrains or hinders the editing machinery. It also indicates that, unlike the gRNA-independent reaction, the A-U element on the mRNA is not essential for the directed insertions. Paradoxically, A to C substitution mutations within the mRNA A-U element are inhibitory to both reactions (Ref. 9, and data not shown). It is possible that the substitutions could have a repulsive steric effect on the editing machinery that is in the vicinity of the editing sites, or, alternatively, the substitutions may result in an inhibitory RNA conformation.

The gRNA-independent and Directed U-insertion Activities

FIG. 3. Mutations to the A-U element on the mRNA affect editing. A, a cytochrome b mRNA transcript containing a deletion of the 34 nucleotide A-U element (−AU, lanes 2–4) was compared with the transcript containing the wild-type A-U element sequence (+AU, lanes 5–7) in the gRNA-directed reaction. The B1 + 2 + 3 gRNA with 3 guiding nucleotides for the first editing site was used for both sets of reactions. The +3 marker indicates the position of correctly edited transcript. ar, artifact. B, quantitation of the reactions in A. Deletion of the A-U element from the cytochrome b mRNA transcript increased the percentage of correctly edited transcripts (+3).

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The mitochondrial extract was also partitioned through a sucrose gradient and both the gRNA-independent (Fig. 4C, top) and the gRNA-directed (Fig. 4C, bottom) activities peak in gradient fractions 5 and 6. As described previously (9), each of the peak fractions contains approximately 10% of the total protein. The two U-insertion reactions, then, co-fractionate with two different partitioning methods suggesting that the reactions exploit an overlapping set of catalysts. It remains possible, however, that additional chromatographic enrichment may resolve differences between the two reactions.
The mRNA A-U Element Does Not Function in trans—

Understanding the role of the cytochrome b mRNA A-U sequence during the gRNA-independent reaction may provide insight into its function on the gRNA. The A-U sequence within the 5′-untranslated mRNA sequence has been proposed to be a binding site for a component of the editing machinery (9). This is supported by gel-shift analysis, which indicates that at least one protein within the fractionated editing extract binds to the mRNA A-U sequence, and this binding is inhibited by mRNA mutations that inhibit the gRNA-independent reaction (9). The hypothesis is also consistent with the 34-nucleotide untranslated sequence being able to induce U-insertions when placed within an unrelated mammalian transcript, and also with the majority (85%) of the gRNA-independent insertions within the cytochrome b transcript being localized to either side of the A-U sequence (9).

An alternative hypothesis is that the 5′-untranslated sequence folds back on the pre-edited sequence and effectively acts as a gRNA in cis (8). However, the mRNA A-U sequence does not contain the sequence that normally would anchor the gRNA to the mRNA (Fig. 1, A and B), and in the absence of this anchor sequence the gRNA-mRNA duplex is not stable. Furthermore, artificial sequences that stabilize the hypothetical fold-back interaction affect the location of the gRNA-independent insertions (8), but do not increase the efficiency of the reaction (9). The mRNA in cis hypothesis is also hard to reconcile with the 34-nucleotide untranslated sequence being able to induce U-insertions when placed within an unrelated mammalian transcript, and also with the majority (85%) of the gRNA-independent insertions within the cytochrome b transcript being localized to either side of the A-U sequence (9).

Determining whether the mRNA A-U sequence can stimulate the gRNA-independent reaction in trans provides a more definitive means to distinguish between the two hypotheses. If the A-U sequence functions as a binding site for the editing machinery, it should not be able to activate the independent reaction in trans, whereas the fold-back hypothesis makes the opposite prediction. Deletion of the mRNA A-U sequence from the circular cytochrome b mRNA substrate inhibits 80% of the gRNA-independent U-insertions (Ref. 9; Fig. 5, lanes 4 and 5). As a first approximation, an RNA forming a duplex in cis has a linear 10–40% sucrose gradient were assayed for both gRNA-independent (top) and gRNA-directed (bottom) editing activities, both of which peak in fractions 5 and 6. Reactions with the unfractonated mitochondrial extract are also shown.
could be related to a decreased affinity for an inhibitory component of the editing machinery and the non-competed fraction with the peak editing activity obtained from the Q-Sepharose column (data not shown), and the non-competed wt A-U-containing and triple-block mutated gRNA. One complex, however, is not competed even by a 5-fold molar excess of the mutated gRNA (hash mark).

ΔG° that is 3 kcal/mol more favorable than the same interaction occurring in trans (15). Given this difference, titration of a 20-fold molar excess of a 37-nucleotide RNA containing the A-U element should have been able to induce gRNA-independent insertions within the mRNA substrate lacking the A-U sequence if the fold-back hypothesis is correct (Fig. 5, lanes 6–9). As this did not occur, it strongly suggests that the hypothetical fold-back interaction is not relevant to the mechanism of the gRNA-independent reaction.

The Mutated gRNA Does Not Compete for a Component of the Editing Extract—The high degree of similarity between the A-U sequence of the mRNA and gRNA is suggestive that the sequences may have similar functions. Gel-shift analysis previously identified multiple protein complexes that form when a 37-nucleotide transcript containing the mRNA A-U element is incubated with the peak sucrose gradient fraction supporting the gRNA-independent reaction (Ref. 9; Fig. 6, lane 1). The A to C substitutions within the mRNA A-U element were shown to inhibit the formation of several of these complexes as well as the gRNA-independent reaction (9). We were interested in determining whether a protein in the extract that binds to the mRNA A-U element also interacts with the A-U sequence of the wild-type gRNA and whether this interaction could be related to the decreased efficiency of the directed reaction. Addition of a competitor consisting of either unlabeled wild-type (wt) A-U-containing and triple-block mutated gRNA. One complex, however, is not competed even by a 5-fold molar excess of the mutated gRNA (hash mark).

Machinery in Vivo—Since the A-U sequence on both the gRNA and mRNA affects the in vitro gRNA-directed reaction, it was of interest to determine whether this sequence also influences the in vivo reaction. The in vitro gRNA-independent U-insertions occur in specific regions of the mRNA and are suggestive of the editing machinery interacting with the mRNA A-U element (9). Many of the in vitro gRNA-independent U-insertions are within the 5′-untranslated region flanking the ends of the mRNA A-U sequence and do not overlap sites of known gRNA-directed editing. Editing detected in vivo at these specific sites, then, could not readily be attributed to gRNA-directed editing. As a result, an in vivo pattern of cytochrome b mRNA mis-editing characteristic of the in vitro gRNA-independent reaction could be suggestive that this interaction also occurs in vivo.

To determine whether non-directed modifications occur in vivo that are indicative of the editing machinery interacting with the mRNA A-U sequence, the 5′-untranslated region from mitochondrial cytochrome b mRNA was cloned and sequenced. Total mitochondrial RNA was isolated from a late log phase L. tarentolae culture and cDNA synthesized using a DNA primer complementary to a sequence 3′ of the cytochrome b editing sites. The full-length cDNA was gel-purified, terminal deoxynucleotidyltransferase was used to add a poly(dA) tail to the 3′ end of the cDNA, and the product was PCR-amplified for cloning and sequencing. Of the 49 clones sequenced, 26 contained U-deletions that could not be attributed to gRNA-directed editing (Fig. 7). Similar results were obtained from the cloning of the 5′-untranslated cytochrome b mRNA sequence from mitochondrial RNA prepared from a mid-log phase culture. To ensure the deletions were not the result of a cloning artifact, the same procedure was used to clone the 5′-untranslated sequence from a synthetic cytochrome b mRNA transcript. None of 17 sequenced clones contained the U-deletions. The genomic sequence encoding the 5′-untranslated region of the cytochrome b was also cloned and sequenced, and none of the 15 sequenced clones contained deletions, making it unlikely that maxicircle heterogeneity can account for the U-deletions.

The U-deletions detected within the cytochrome b mRNA are restricted to two regions of the 5′-untranslated sequence, at these specific sites, and non-gRNA-directed U-deletions are indicated. The location of the 5′-most gRNA-directed editing site is also indicated.
The 5′ end of the ND7 mRNA was also cloned from the mitochondrial RNA, but no U-deletions were detected in 17 sequenced clones. This further emphasizes that the observed cytochrome b mRNA deletions are not a cloning artifact and is suggestive that the modifications maybe unique to the cytochrome b mRNA. The maxicircle sequence immediately upstream of the ND7 coding sequence contains a 16 out of 18 nucleotide match with the cytochrome b mRNA A-U element (9). Only 12 matched nucleotides, however, are present on the 5′ end of the mature ND7 transcript (16), and this may not be sufficient to function in vivo in the same manner as the cytochrome b sequence.

Non-gRNA-directed U-insertions were not detected within the mitochondrial cytochrome b sequence, even when the cDNA used for cloning had been size-selected to enrich for insertions. This was unexpected given the detection of in vitro gRNA-independent U-insertions. However, the in vitro gRNA-independent U-insertions could only be localized within individual RNAs after considerable enrichment exploiting thiouridine incorporation and organomercurial partitioning (9). Because there was no corresponding enrichment for RNAs with deletions, it is possible that non-directed U-deletions also occur in vitro. Furthermore, at least in T. brucei, the U-deletion and -insertion activities co-purify within the same complex and are differentially optimized (17). As a result, it is also possible that if the complex is associated with the mRNA A-U element, deletions could be the predominant in vivo editing activity.

**DISCUSSION**

Two similar A-U sequences located within the 5′-untranslated region of the cytochrome b mRNA and within the cytochrome b gRNA I significantly affect in vitro editing. The mRNA A-U sequence is necessary and sufficient to induce U-insertions independent of gRNA (9). In contrast, significant guiding of in vitro U-insertions does not occur unless the cytochrome b gRNA A-U sequence has been mutated. This gRNA-directed editing of a cytochrome b transcript was demonstrated with a primer extension assay and confirmed through sequencing (Fig. 2). Thus, a sequence element, originally identified on the mRNA because it induces gRNA-independent U-insertions, inhibits the directed reaction when present on the gRNA. There are several possible explanations for the mutations to the gRNA A-U sequence being required to activate the gRNA-directed editing reaction. First, it is possible that an interaction of the A-U sequence of the mRNA with that of the gRNA inhibits the reaction; the mutations would function to disrupt the hypothetical interaction. This is unlikely, however, because in vitro editing of mRNA transcripts containing a deleted A-U sequence is still stimulated by a gRNA with a mutated A-U sequence (data not shown). It is also possible that the A-U element of the gRNA could be interacting with a different part of the mRNA. We truncated the 205-nucleotide cytochrome b transcript initially used in the assay to 96 nucleotides and did not detect an effect on the assay (data not shown). Aside from the A-U nucleotide A-U element, the remaining 82 nucleotides of the truncated mRNA transcript are not very A-U-rich and would not be expected to form a highly favorable structure with the gRNA A-U element. In an attempt to disrupt the hypothetical interaction and promote proper annealing of the wild-type gRNA and mRNA, we have used several denaturing and annealing conditions as well as the human immunodeficiency virus nucleocapsid protein, an RNA chaperone, with no positive effect on the assay (data not shown). We could not detect significant conformational differences by native gel electrophoresis between gRNAs containing the wild-type and mutant A-U sequence nor between the different gRNA-mRNA duplexes (data not shown). It is also possible that the directed reaction is inhibited because the gRNA A-U element is interacting with an A-U element of another cytochrome b gRNA. However, native gel electrophoresis failed to detect gRNA dimers (data not shown). In addition, editing efficiency would be expected to increase as the gRNA and mRNA concentrations are reduced because the formation of the hypothetical gRNA dimers would become less favorable, and this also was not observed (data not shown). It is also possible that the gRNA-directed reaction could have been affected by the 4 point mutations introduced into the gRNA binding site of the mRNA transcripts or by the compensatory changes introduced within the gRNAs (Fig. 1B). However, the same activation of guiding was reproduced in the context of the natural gRNA and mRNA sequences although the higher background complicates quantitation (data not shown).

An alternative possibility for the activation of the directed reaction is that factors within the extract bind to the A-U sequence of the gRNA and inhibit editing. The high degree of sequence similarity between the A-U sequence of the mRNA and the gRNA suggests that the two sequences may function similarly. Some of the same mutations to the A-U element of the gRNA that activate the directed reaction also inhibit the independent reaction when made in the context of the mRNA A-U element (Fig. 2; Ref. 9). A relationship between the gRNA-directed and independent activities is further supported by their co-fractionation through both a Q Sepharose column and a sucrose gradient (Fig. 4). The role of the mRNA A-U element is most consistent with a model in which it binds to a protein that either interacts with or is a component of the editing machinery (Fig. 5; Ref. 9). This suggests that a protein that is required for the gRNA-independent activity may also interact with the gRNA A-U element and inhibit the directed reaction. We have not been able to fractionate the hypothesized inhibitor away from the editing activity using either the Q Sepharose column or sucrose gradient, which implies that it is more likely to be related to editing rather than being some unrelated protein that interferes with the in vitro reaction (data not shown).

Gel-shift analysis indicates that the gRNA containing the wild-type A-U element effectively competes for the same proteins that form complexes with the mRNA A-U sequence (Fig. 6). However, the A to C mutated gRNA failed to compete with the mRNA A-U sequence for the formation of one of the complexes. The same mutations to the mRNA A-U sequence have previously been demonstrated to inhibit the formation of several complexes as well as the gRNA-independent reaction (9). Thus, the formation of the non-competed complex on the mRNA correlates with the gRNA-independent U-insertion activity, whereas its presence on the gRNA correlates with inhibition of the gRNA-directed reaction.

The high number of U-deletions detected in the cytochrome b mRNA isolated from the mitochondria (Fig. 7) is suggestive that an interaction between the editing machinery and the mRNA A-U element may also occur in vivo. Although we cannot completely eliminate the possibility that the non-genomically encoded deletions are caused by gRNAs, three observations would argue against this. First, most of the gRNAs in the L. tarentolae UC strain have been identified (18), and none of these encode for the observed deletions. In addition, more than one gRNA would probably be required to produce the observed bipartite distribution of U-deletions (Fig. 7). Second, gRNA-mRNA chimeras were not detected within the mRNA A-U element, as might have been expected if the deletions were...
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caused by a gRNA-directed process. Third, the deletions are restricted to the sequence immediately flanking the A-U sequence, as predicted by the in vitro results. We did not find any evidence of non-canonical editing outside of this region, as would have been expected if there was a high frequency of misguiding events (19). This is consistent with the possibility that the A-U sequence of the cytochrome b mRNA is serving as a binding site for the editing machinery and the adjacent sequence is a substrate for both in vitro and in vivo modifications.

The 5′-untranslated sequence of the cytochrome b mRNA had been previously determined by reverse-transcriptase sequencing of poly(A) ‡ RNA isolated from the mitochondria, and deletions were not reported (7). However, individual clones were not sequenced, and since less than 50% of the clones contained deletions at any given position, the deletions would not have been readily apparent from the poly(A) ‡ RNA sequence.

There are several potential benefits to localizing the editing machinery on discrete sites of a mRNA or gRNA. First, it would be a means through which the machinery could be preferentially concentrated near messages that need to be efficiently edited. Second, it could sequester the editing machinery, preventing reactions at inappropriate sites. An unpaired nucleotide at the end of a duplex is the RNA structural feature believed to define the location of a gRNA-mediated editing site (2, 3, 20). The mitochondrial mRNAs would be expected to contain several intramolecular helices that could serve as fortuitous substrates for the editing reaction. Sequestering the editing machinery at discreet sites would be one means through which inappropriate editing could be limited. The mRNA A-U sequence could also function as a transitory binding site for the editing machinery. As editing proceeds, the gRNA is predicted to become duplexed with the mRNA masking the presumed recognition element. The mRNA A-U element could function to keep the editing machinery in the vicinity of a partially edited mRNA until another gRNA, guiding subsequent sites, is appropriately positioned. In addition, the A-U element could be a means through which the editing reaction is regulated. There are developmental stages of T. brucei during which specific pre-edited mRNAs are not edited even though the appropriate gRNA is present (21–23). The expression of factors that release the editing machinery from specific gRNAs or mRNAs could be one possible mechanism through which this regulation is achieved. The mitochondrial genome is very A-U rich, and it is possible that there are permutations of the cytochrome b mRNA A-U element that would be able to perform similar functions on other RNAs. Alternatively, the effects mediated by the A-U element may be unique to the cytochrome b mRNA and gRNA.

Acknowledgments—We are grateful to K. Musier-Forsyth, H. Meehan, and H. Hiasa for helpful suggestions during the course of this work.

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