A Cis-acting A-U Sequence Element Induces Kinetoplastid U-insertions

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A 34-nucleotide A-U sequence located immediately upstream of the editing sites of the Leishmania tarentolae cytochrome b mRNA induces a mitochondrial extract to insert U nucleotides independent of guide RNA. Insertions are localized to positions immediately 5' and 3' of the A-U sequence. When placed within an unedited mammalian transcript, the A-U sequence is sufficient to induce U-insertions. The sequence has a high degree of similarity with the templating nucleotides of a cytochrome b guide RNA and with a sequence adjacent to the editing sites in ND7 mRNA, the other characterized kinetoplastid mRNA supporting guide RNA-independent U-insertions. At least one protein specifically interacts with the A-U sequence. The reaction is consistent with a mechanism proposed for guide RNA-directed editing.

Several mitochondrial mRNAs of the kinetoplastid protozoa must be edited through the precise insertion and deletion of U nucleotides (reviewed by Refs. 1–3). The editing has been proposed to proceed through a reaction that is initiated at an editing site by an endonucleolytic cleavage producing a 5'-fragment with a 3'-OH and a 3'-fragment with a 5'-monophosphate (4). U-addition by a terminal uridylyltransferase activity (TUTase) or U-deletion by an exonucleolytic activity at the 3'-OH followed by re-ligation of the 5'- and 3'-fragments would complete one editing cycle which could be reiterated at subsequent sites. Experimental evidence in support of the proposed mechanism has been obtained in vitro (5–8).

Genetic information specifying the location of the U-insertions and deletions is carried on guide RNAs (gRNAs). A specific gRNA is able to bind to its cognate mRNA immediately 3' of a block of editing sites and also contains a template complementary to a block of edited sequence (4). It has been demonstrated in vitro for certain Trypanosoma brucei and Leishmania tarentolae mRNAs that the appropriate gRNA directs the U-insertions and deletions at the editing sites; changing the templating sequence of the gRNA results in the corresponding change to the number of U-insertions and deletions (5–8).

U-insertions within some L. tarentolae mRNAs have been reported to be catalyzed by a mitochondrial lysis independent of added gRNA. Initially it was suggested that gRNAs endogenous to the mitochondrial lysis were directing the insertions (9). However, it was subsequently demonstrated that the U-insertions were unaffected by mutations to the mRNA that should have inhibited interactions with endogenous gRNA (10). Characterization of the reaction was complicated by difficulty in solubilizing the majority of the U-insertion activity from the crude mitochondrial lysis, making subsequent enrichment inefficient and leaving the assays vulnerable to artifacts caused by contaminating activities. The low level of U-insertions also hindered any significant biochemical characterization and prevented the localization of the U-insertions within individual RNAs. As a result, the biological significance of the gRNA-independent reaction observed in vitro was unclear.

We describe here a novel assay that has facilitated the enrichment and characterization of the gRNA-independent U-insertion activity. The majority of the U-insertions occurring independent of gRNA are adjacent to a 34-nucleotide A-U sequence element that is immediately 5' of the editing sites on the cytochrome b transcript. This element is both necessary for the cytochrome b gRNA-independent editing and also sufficient to induce gRNA-independent insertions within a mammalian transcript. The sequence of the A-U element is highly similar to the templating sequence of cytochrome b gRNA I and a sequence immediately upstream of editing sites of the ND7 mRNA, which is the other characterized mRNA that supports gRNA-independent U-insertions (8). The intermediates produced during the reaction are consistent with a mechanism proposed for gRNA-directed editing (4). Our results suggest that gRNA-independent editing may result from association of the editing components with the 34-nucleotide A-U element present on the cytochrome b mRNA.

EXPERIMENTAL PROCEDURES

Mitochondrial Extract Preparation—An old laboratory strain (UC strain) of L. tarentolae was grown to a density of 1 to 2 × 10⁸ cells/ml in 2-liter flasks containing 500 ml of BHI media (DIFCO) supplemented with 10 μg/ml hemin. The flasks were loosely covered with foil and shaken at 100 rpm at 27 °C in an Innova 4230 incubator (New Brunswick Scientific). After washing three times with SHE buffer, cells were lysed in a Stansted Disrupter (Energy Service Co.) at 1200 p.s.i. as described previously (10). Mitochondria were purified by flotation in a Renograffin density gradient (11), and after washing three times with SHE, resuspended in 950 μl of solubilization buffer (25 mM Hepes 7.5, 10 mM MgCl₂, 1 mM KCl, 0.1 mM ATP, 1 mg/ml Pefabloc, and 10 μg/ml leupeptin) for every 500 ml of starting culture. Triton X-100 was added to a final concentration of 0.5%, and the solution was gently mixed by inversion and left on ice for 5 min. The mitochondrial lysate was centrifuged at 11,000 × g at 4 °C for 5 min and the supernatant frozen on dry ice for storage at −80 °C. The protein concentration of the unfractionated extract was 2.3 ± 0.1 μg/μl. A 250-μl aliquot of the extract was thawed on ice and loaded onto a linear 5–10% sucrose gradient and centrifuged in a Ti55 rotor at 100,000 × g for 12 h at 4 °C. Fractions (450 μl) were collected from the top of the gradient.

RNA Transcripts—RNAs were transcribed either from PCR products or directly from synthetic oligodeoxynucleotides using T7 RNA polymerase (12). The template for the parental transcript (wild type) contain-
ing the 5′-178 nucleotides of pre-edited cytochrome b and a mutated gRNA-binding site was amplified from plasmid pNB2-S1 (9) using oligodeoxynucleotide primers 5692 and 10211. The same plasmid was used both for the amplification of the Δ(3–10) template with primers 10513 and 10211, and the S(8A to 8C) template with 10703 and 10211. The transcript used to demonstrate the importance of a 5′-phosphate and 3′-OH to the ligation reaction was synthesized by RT-PCR of circular wild type cytochrome b using primer 10076 followed by PCR amplification of the extension products using 10076 and 10132. The template for Δ(31–39) was similarly synthesized by RT-PCR using 10795 and 10796. The templates for the 5′-extended wild type and S(8A to 8C) cytochrome b RNAs were synthesized by RT-PCR of circular wild type cytochrome b (Δ(31–39)) and circular S(8A to 8C) RNAs, reverse transcribed followed by ethanol precipitation. Products were resolved on 9% denaturing polyacrylamide gels and quantified after PhosphorImager scanning (Molecular Dynamics). 

Enrichment of RNAs with grnNA-independent U-insertions—Circular cytochrome b transcripts were extract treated under the direct assay conditions with 1 mm 4-thio-UTP (Amersham) substituted for both labeled and unlabeled UTP. Circular RNAs were purified on 6% denaturing polyacrylamide gels, eluted, and ethanol precipitated. RNAs were resuspended in a total volume of 50 μl containing 0.5% SDS and 6 μM EDTA. After heating at 65 °C for 3 min, the RNA solution was added to a 1.5-ml plastic tube containing 10 μl of oligomercury-derivatized agarose (Bio-Rad) that had been washed three times in 1× buffer (400 mm NaCl, 40 mM Hepes pH 7.5, 3 μM EDTA, and 0.5% SDS) and 25 μl of 0.5% SDS. The RNA was incubated at 27 °C for 30 min on a rocker. The bead were pelleted for 5 s in a microcentrifuge and after removing the supernatant, resuspended in 600 μl of 1× buffer and heated at 65 °C for 5 min. The beads were re-pelleted, the supernatant discarded and the washing procedure repeated an additional 5 times. After the last wash, RNA was eluted from the beads by heating at 65 °C in 1× buffer containing 100 mm dithiothreitol and 50 pmol of the DNA primer (7534 or 10040) used for subsequent CDNA synthesis. The RNA was ethanol precipitated using 5 μg of glycogen (Boehringer-Mannheim) as a carrier, and the oligomercury column enrichment procedure repeated an additional two times. The RNA from the 3rd cycle of enrichment was reverse transcribed into CDNA and PCR amplified for cloning using primers 7534 and 8494 or 10040 and 10041. Bio-X-ACT (Novagen) was used to increase the fidelity of the amplification.

Other Assays—U-insertions within extract-treated linear RNAs were detected using an RNase H reaction (9). Linear RNAs were extract treated as described for the circular substrates with the exception that the UTP concentration was at 0.5 μM and the specific activity increased to 3000 Ci/mmol. Hepes buffer (100 mM, pH 7.4) was included within the reactions to reduce the amount of 3′-end labeling relative to the internal U-adoptions. The extract reactions were terminated by the addition of EDTA to a final concentration of 20 μM and incubation at 27 °C for 3 min followed by the addition of SDS to 0.5%, proteinase K (50 μg/ml), and a further 30-min incubation. The RNA was then phenol/chloroform extracted, gel purified, eluted, and ethanol precipitated. Oligodeoxynucleotide (ODN) gels were used to separate radiolabeled bands. Several criteria were used to identify and purify the circular RNAs from intermolecular ligation products potentially complicating the U-insertion assay. First, primer extension analysis was used to extend through the ligation junction. Extension of a circular RNA yields a CDNA product that is close to full-length whereas extension of an intermolecular dimer yields both this CDNA and, providing the RT primer-binding site is not at the extreme ends of the RNA, a shorter second product. Second, when radiolabeled GTP primed and unlabeled GMP primed RNAs are incubated in a ligase reaction followed by primer extension the nucleotides are extended from the labeled 5′ end of the RNA. The bands identified by the primer extension, are the only predominant radiolabeled bands. Third, the electrophoretic mobility of circular RNAs relative to linear RNAs varies significantly with the percentage of acrylamide. Small quantities of linear contaminants co-migrating with circular RNAs on the 6% gel are readily detected when re-electrophoresed on higher percentage gels.
Clones with the same mapped cleavage site were included only if the length of each poly(C) extension was unique indicating that they had originated from different RNAs. There is a 1 nucleotide uncertainty in mapping the cleavage sites adjacent to genomically encoded C nucleotides, and when UTP is included within the editing reaction, the mapping is also complicated by genomically encoded U nucleotides.

**Northern Blot Analysis—**RNA isolated from the sucrose fractions was electrophoresed in a 1.2% agarose/formaldehyde gel and transferred to Hybond N+ membrane (Amersham). Hybridization in Church-Gilbert buffer (0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS, 1% bovine serum albumin) was performed at 65 °C for the 12 S probe (oligodeoxynucleotide 10855) and at 55 °C for the gRNA probe (oligodeoxynucleotide 10854). Oligodeoxynucleotide probes were 5’-end labeled and were added to the hybridizations at a final concentration of 1 × 106 cpm/ml and a specific activity of 1 × 109 cpm/ml.

**Oligodeoxynucleotides—**Oligodeoxynucleotides shown below were used in this study. The locations of the underlined sequences within either the L. tarentolae maxicircle sequence (GenBank entry LEIKPMAX) or the sequence of an EST encoding the human ferritin light chain (DNA Sequence accession numbers AA23712 and EST76262) are indicated. 5690, TAATACGACTCACTATA. 5692, TAATACGACTCACTATAGGGACCATCTTCTCAGCCTGCAGGTACCCTATAAACGGTGCTGGCAGGTCC (nt 142–160 in AA323712). 10076, TATAATTATTTAAAATTTAAATTAAATTTATCCTATAGTGAGTCGTATTA (nt 259–296 in AA323712). 10211, ACAAATAAAGCAACTAAAAAATAATTATATAATG. 10313, TAATACGACTCACTATAGGGACAATTGAAGTTCA. 10403, CTTTTATAATTAGGGAAAAGGGAAAGGAAATTAAATTTATCCTATAGTGAGTCGTATTA (nt 5371–5408 in LEIKPMAX). 10513, TAATACGACTCACTATAGGGATTTATTATAATTTATAATTAAAATTTAATTTAAAATTTATCCTATAGTGAGTCGTATTA (nt 5371–5408 in LEIKPMAX). 10703, TAATACGACTCACTATAGGGATTTATTATAATTTATAATTAAAATTTAATTTAAAATTTATCCTATAGTGAGTCGTATTA (nt 5371–5408 in LEIKPMAX). 10704, TAATACGACTCACTATAGGGATAAATTTCCAGGAGGTAGG (nt 279–301 in AA323712)....
The non-permuted cytochrome c is circularly permuted by RNA polymerase. This resulted in the generation of molecules with U-insertions to also be detected by RT-PCR. The specific activity of the gRNA-independent U-insertion activity in fractions 6 and 7 is increased compared to the unfractionated extract. The specificity of the gRNA-independent U-insertion activity in fractions 6 and 7 is increased compared to the unfractionated extract. The specific activity of the gRNA-independent U-insertion activity in fractions 6 and 7 is increased compared to the unfractionated extract. The specific activity of the gRNA-independent U-insertion activity in fractions 6 and 7 is increased compared to the unfractionated extract.

The endogenous cytochrome b gRNA I is spread throughout the gradient (Fig. 1A, Northern analysis). The wide distribution is suggestive that the gRNA is in heterogeneously sized complexes with other factors, in agreement with previous studies (18–21). The fractions containing the peak U-insertion activity (6 and 7) each contain approximately 10% of the total protein loaded onto the gradient. The remaining increase in specific activity is probably a result of fractionating away RNAs or proteins that are inhibitory to the reaction.

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The U-Insertions Are Not Random—The low efficiency of the gRNA-independent reaction had previously prevented the cloning and sequencing of individual RNAs with insertions (9). We enriched for RNAs containing inserted U nucleotides by exploiting the observation that 4-thiouridine could be incorporated into the cytochrome b transcript with 85% of the efficiency of uridine (data not shown). RNAs containing 4-thiouridine insertions form mercaptides with an organomercurial column matrix which permit their partitioning from the bulk un-modified RNA (Fig. 2A). Those RNAs containing thiouridine insertions were eluted from the organomercurial matrix with dithiothreitol for RT-PCR, cloning, and sequencing.

After organomercury column enrichment, 28% of the sequenced clones contained inserted U nucleotides (Fig. 2, B and C). As a result of the assay design, only 108 nucleotides of the substrate RNA could initially be screened for insertions (arrows in Fig. 2C); the remaining 70 nucleotides were used as primer-binding sites during the RT-PCR amplification and any U-insertions within this region would not have been detected. In a parallel experiment, however, primers binding to different regions of the circular cytochrome b RNA were used for the RT-PCR. This resulted in the generation of molecules with circularly permuted ends and permitted U-insertions to also be detected within the original primer-binding sites (circles in Fig. 2C). The U-insertions are predominantly localized either within an 8-nucleotide sequence corresponding to the 5'-end of the non-permuted cytochrome b transcript or within an 8-nucleotide region that is normally edited in vivo (Fig. 2C, boxed sequence). It should be emphasized that there are no insertions between nucleotides 9 and 34 and only rare insertions within the region 3' of the first editing site (nucleotides 58–178). Thus, the non-permuted cytochrome b gRNA-binding site still results in U-insertions within editing sites, although with less accuracy than occurs in vivo (Fig. 2C, bottom panel). All clones contained the mutagenized gRNA-binding site eliminating any possibility that they had been derived from endogenous mRNA. Insertions that occurred adjacent to genomically encoded U nucleotides could not be precisely localized (Fig. 2C, positions 8, 108, and 128).

The observed U-insertions are not the result of either a PCR or cloning artifact based on the following observations. First, clones containing inserted U nucleotides were only detected if the RNAs were enriched by passage through the organomercury column prior to RT-PCR and cloning. When the extract-treated RNAs were amplified and cloned without sufficient enrichment, none of 60 sequenced clones had insertions. Second, the insertions within all of the sequenced clones were restricted to U nucleotides. If the U-insertions had resulted from errors during the PCR amplification or cloning, the insertion of other nucleotides would have been expected.

Nine of the cloned RNAs that were enriched for U-insertions also contain deletions. These deletions are preferentially localized either at U-nucleotides or adjacent to inserted U-nucleotides (data not shown).

An A-U Sequence Element Immediately Adjacent to the 5'-Most Editing Site Is Necessary for the gRNA-independent U-insertions—Since the editing sites of most pre-edited mRNAs are rich in purines, it was initially assumed that the purines may serve as a recognition site for the U-insertion machinery, accounting for the predominance of gRNA-independent insertions within this region (Fig. 2C, boxed sequence). However, deletion of the purine-rich pre-edited region (Fig. 3A) reduced the reaction by a factor of 1.7 ± 0.3 (mean from six reactions). As already indicated (Fig. 1), modification of the gRNA-binding site has no significant effect on the reaction efficiency. Deletions made 3' of the gRNA-binding site also have no effect (data not shown).

The U-insertions, instead, are dependent on a 34-nucleotide A-U sequence that is within the untranslated region immediately 5' of the in vivo editing sites (Fig. 3A). Deletion of 7 nucleotides from the 5'-end of this sequence resulted in the loss of 82 ± 8% (mean from six reactions) of the gRNA-independent insertions (Fig. 3B, lane 2), and deletion of 7 nucleotides from the 3' end of the A-U sequence resulted in an 84 ± 4% (mean from six reactions) reduction (Fig. 3B, lane 3). Substitution of eight of the internal A nucleotides with C nucleotides likewise abolished 86 ± 2% (mean from six reactions) of the insertions (Fig. 3B, lane 1). The decreased number of U-insertions within the mutated RNAs is a result of a decreased stability within the mitochondrial extract; incubation of radiolabeled RNA with the mitochondrial extract in the absence of [α-32P]UTP indicated that the S(8A to 8C) mutated RNA is as stable as the wild type (Fig. 3B, lanes 7 and 8).

The A-U sequence is sufficient to induce U-insertions when placed within an unedited mammalian transcript. A 192-nucleotide linear transcript containing part of the coding sequence of mammalian ferritin was treated with the mitochondrial extract; incubation of radiolabeled RNA with the mitochondrial extract in the absence of [α-32P]UTP indicated that the S(8A to 8C) mutated RNA is as stable as the wild type (Fig. 3B, lanes 7 and 8). The A-U sequence is sufficient to induce U-insertions when placed within an unedited mammalian transcript. A 192-nucleotide linear transcript containing part of the coding sequence of mammalian ferritin was treated with the mitochondrial extract; incubation of radiolabeled RNA with the mitochondrial extract in the absence of [α-32P]UTP indicated that the S(8A to 8C) mutated RNA is as stable as the wild type (Fig. 3B, lanes 7 and 8).
nucleotide element (S(8A to 8C), see also Fig. 3A) of the chimeric transcript inhibited the induction of internal U-insertions (Fig. 4A, lane 8) but did not inhibit the additions to the 3'-end (Fig. 4A, lane 7). This is consistent with the cytochrome b RNA mutagenesis (Fig. 3B, lane 1) and suggests that the U-insertions are induced directly by the A-U element rather than being an indirect consequence of the 34-nucleotide substitution. It is possible, however, that the stimulation of 3'-end labeling observed with both the wild type A-U and the 8A to 8C substitutions are the result of such an indirect effect.

A sequence extension on the 5'-end of the A-U element significantly enhances U-insertions into the linear cytochrome b transcript but is not absolutely required. The efficiency of U-insertions within the linear 178-nucleotide cytochrome b transcript (Fig. 4B, lanes 3 and 4) is decreased approximately 15-fold relative to the corresponding circular RNA (data not shown). The 8A to 8C substitution within the linear transcript (Fig. 4B, lanes 5 and 6) reduced the U-insertions by a further factor of 2.0 ± 0.4 (mean from 8 reactions), suggesting that approximately half the U-insertions within the linear transcript are still dependent on the A-U element (Fig. 4A, lanes 3 and 4). If 60 nucleotides are added to the 5'-end of linear
cytochrome b transcript through circular permutation (22), the level of insertions approximates that of the corresponding circular RNA (Fig. 4A, lanes 7 and 8). Thus, the greater efficiency of U-insertion detected with the circular RNA is probably a consequence of adding nucleotides to the 5'-end of the A-U element and not an indirect effect of the circularization. The 8A to 8C substitution within the A-U element of the 5'-extended transcript inhibits the U-insertions (Fig. 4B, lanes 9 and 10). This is consistent with the effect of the same mutation on U-insertions within the circular RNA (Fig. 3B, lane 1).

There Is Specific Binding of the A-U Element to at Least One Protein within the Fractionated Mitochondrial Extract—Gel-shift analysis was used to detect factors within the fractionated mitochondrial extract that specifically interact with the A-U sequence. Incubation of sucrose gradient fractions 6 and 7, the peak U-insertion fractions (Fig. 1A), with a 37-nucleotide transcript containing the A-U element results in formation of 6 complexes (Fig. 5, lane 2, A-F). Four of the complexes (labeled A, B, D, and E) are not detected when radiolabeled 8A to 8C substituted RNA is used in the assay (lane 1). These four bands are sensitive to pretreatment of the extract with proteinase K (data not shown). Since the same 8A to 8C substitutions also inhibited U-insertions within the cytochrome b transcripts (Fig. 3, lane 1), the missing complexes (A, B, D, and E) could be directly relevant to the U-insertion reaction. A greater amount of complex C forms with the 8A to 8C substituted RNA than with the wild type A-U transcript. This is suggestive that complex C could possibly be a precursor to the formation of complex E which is inhibited by the same mutation.

Three of the bands shifted by the A-U element can be competed by an unlabeled 41-nucleotide ferritin transcript (Fig. 5, lanes 3–9). Whereas complexes A, B, and D are efficiently competed by the inclusion of low levels of the ferritin RNA during the binding reaction, bands C, E, and F are only competed approximately 50% by a 10-fold molar excess. The low level of U-insertion detected within the 192-nucleotide linear ferritin transcript (Fig. 4A, lanes 3 and 4) and in other transcripts not containing the A-U element (Fig. 3B, lanes 1–3) suggests that the U-insertion machinery can also interact relatively nonspecifically with RNA. It seems probable, however, that the induction of U-insertions when the A-U element is present involves complex E as it is both sensitive to the same A to C substitutions that inhibit U-insertions and is also relatively resistant to competition by the ferritin transcript. The $K_d$ for this complex is approximately 200 nm.

The gRNA-independent U-insertion Reaction Is Consistent with a Mechanism Proposed for gRNA-directed Editing—Previous studies have suggested that gRNA-directed editing proceeds through a mechanism involving sequential endonucleolytic cleavage at an editing site followed by the addition of uridine nucleotides to the 3'-OH group of the 5'-fragment and re-ligation of the fragments (23). For the in vitro editing of the T. brucei ATPase 6 mRNA, the best characterized in vitro kinetoplastid editing system, the U-additions and deletions are dependent upon the addition of gRNA (24, 25). If the U-insertion components can assemble on some L. tarentolae pre-edited mRNAs independent of gRNA, an analogous mechanism could be mediating the gRNA-independent reaction. Such an assembly of U-insertion components on the pre-edited mRNA is supported by the gel-shift analysis (Fig. 5).

Consistent with a cleavage/U-addition/ligation mechanism, an RNA ligase is present within the same sucrose gradient...
fractions as the gRNA-independent U-insertion activity (Fig. 6A, lanes 1–11). Fractions 6 and 7, the peak U-insertion fractions (Fig. 1A), efficiently ligate radiolabeled linear cytochrome b RNAs containing a 3'-OH and 5'-monophosphate in a similar manner to T4 RNA ligase; linear dimers and intramolecular circles are formed in both reactions (Fig. 6A, lanes 6 and 7). The relative concentration of dimer and intramolecular ligation products is dependent upon the RNA substrate concentration and the sequence of the RNA used in the reaction. The reactions were performed in the presence of ATP and UTP under the same conditions in which the U-insertions occur. RNAs with a 3'-monophosphate and a 5'-OH are not substrates for the mitochondrial ligase activity (Fig. 6B), suggesting that if the reaction does proceed through a cleavage/ligation mechanism, it would require that a 3'-OH be produced during the endonucleolytic cleavage. The peak fraction (Fig. 6A, lane 6) has a low level of ligase activity in the absence of added ATP which probably results from ligases that had been adenylated prior to the fractionation (Fig. 6A, lane 12). Similar to some other RNA ligases (26), the kinetoplastid ligase can also utilize UTP (Fig. 6A, lane 13). The presence of both ATP and UTP stimulates the reaction 6-fold relative to UTP alone (Fig. 6A, compare lanes 6 and 13). Kinetoplastid RNA ligases have previously been described (27–30).

Intermediates were isolated from the gRNA-independent reaction that are consistent with a cleavage/U-addition/ligation reaction mechanism. Endonucleolytic cleavage of the circular cytochrome b transcript during the U-insertion reaction produces full-length linear RNAs. The full-length linear molecules formed during the U-insertion reaction were gel purified and a poly(C) extension was added by poly(A) polymerase. The extension products were amplified by cloning by RT-PCR using a poly(G) primer and a primer containing the sequence of the mutated gRNA-binding site. The position at which the circular RNA was cleaved within the extract could then be ascertained by determining the sequence immediately adjacent to the poly(C) extension (Fig. 6C). Under the U-insertion reaction conditions, the sites of RNA cleavage do not correspond well with the sites of U-insertion (filled circles, Fig. 6C). Since the gRNA-independent U-insertion machinery appears to be part of a complex (Fig. 1A), it was reasoned that those RNAs cleaved within the extract as part of the editing reaction would be rapidly re-ligated back to circular RNAs, whereas those that are cleaved by contaminating nucleases would accumulate at a faster rate. In fact, when NTPs are excluded from the reaction so as to inhibit the ligase (Fig. 6A, −NTP), the sites of gRNA-independent cleavage in 50% of the sequenced RNAs now correspond with the sites of gRNA-independent U-insertion (Fig. 2C). If the RNA ligase is inhibited through the exclusion of NTP from the reaction, the sites of cleavage (open circles) in 50% of the sequenced molecules now correspond to the sites of U-addition.

Intermediates of the gRNA-independent reaction were isolated by using sucrose density gradients. There are several RNA-protein complexes form upon incubating the fractionated mitochondrial extract (fractions 6 and 7 pooled) with a 37-nucleotide transcript containing the 34-nucleotide A-U element (lane 2) or the 8A to 8C substitution (lane 1). An unlabeled ferritin transcript was added as competitor with the wild type RNA in lanes 3–8. As a control, the wild type sequence was also incubated in the absence of extract (lane 9).

**Fig. 5.** The A-U element specifically binds at least one protein within the fractionated mitochondrial extract. Several RNA-protein complexes form upon incubating the fractionated mitochondrial extract (fractions 6 and 7 pooled) with a 37-nucleotide transcript containing the 34-nucleotide A-U element (lane 2) or the 8A to 8C substitution (lane 1). An unlabeled ferritin transcript was added as competitor with the wild type RNA in lanes 3–8. As a control, the wild type sequence was also incubated in the absence of extract (lane 9).

**Fig. 6.** The gRNA-independent U-insertion reaction is consistent with the mechanism proposed for gRNA-directed editing. A, an RNA ligase co-fractionates with the U-insertion activity. Linear 32P-labeled cytochrome b transcript containing a 5'-monophosphate and a 3'-OH group was incubated with the sucrose gradient fractions. There is little ligation in the absence of added NTP (−NTP, lane 12). UTP can support the reaction (+UTP, lane 13). B, although linear cytochrome b RNAs containing a 3'-OH and a 5'-monophosphate are chased into circular products by the fractionated mitochondria extract, RNAs with a 3'-monophosphate and a 5'-OH are not. C, under the U-insertion reaction conditions, the sites of endonucleolytic cleavage (filled circles) are not within the in vivo edited region (boxed) and do not correspond well with the sites of gRNA-independent U-insertion (Fig. 2C). If the RNA ligase is inhibited through the exclusion of NTP from the reaction, the sites of cleavage (open circles) in 50% of the sequenced molecules now correspond to the sites of U-addition.
DISCUSSION

A novel direct assay was developed that permitted the enrichment and characterization of gRNA-independent U-insertions. We have found that these insertions are dependent on a 34-nucleotide A-U element that is located within the 5'-untranslated sequence immediately adjacent to the editing sites. The U-insertions are preferentially localized both 5' and 3' of the A-U sequence. Previously, greater than 95% of the U-insertions within a population of extract-treated linear cytochrome b RNAs were indirectly mapped to the pre-edited region using an RNase H-based assay (9). The localization of the U-insertions (Fig. 2C) combined with the results from the deletion and substitution mutagenesis (Fig. 3), the induction of U-insertions within a mammalian ferritin transcript (Fig. 4A), and the gel-shift assay (Fig. 5) collectively point to the A-U element being a binding site for at least one component of the U-insertion machinery.

The L. tarentolae maxicircle sequence was searched for similarity to the A-U element. There is a 20-nucleotide match of the A-U element to a 22-nucleotide sequence of cytochrome b gRNA I (Fig. 7A). The 8A to 8C substitutions that reduce nearly 90% of the gRNA-independent U-insertions (Fig. 3, lane 1) do not support the formation of complexes A, B, D, and E (Fig. 5, lane 1) and inhibit the induction of U-insertions within the ferritin mRNA (Fig. 4A) and the gel-shift assay (Fig. 5) collectively point to the A-U element being a binding site for at least one component of the U-insertion machinery.

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A biological role for the A-U element. A, the L. tarentolae maxicircle was searched for sequences containing the A-U element. There is a 20-nucleotide match of the A-U element to a 22-nucleotide sequence of cytochrome b gRNA I (nucleotides 16760–16781 in LEIKPMAX) and a 16-nucleotide match within an 18-nucleotide sequence immediately 5' of the editing sites in the mRNA encoding ND7 (nucleotides 3283–3299 in LEIKPMAX). Vertical lines indicate identical bases. The location of the 8A to 8C substitutions that inhibit over 80% of the U-insertions are also indicated. Cytochrome b gRNA I is shown below base pairing with the pre-edited mRNA (bottom). Lowercase letters represent the templating nucleotides. The sequence of the gRNA that matches that of the A-U element is shaded and the first 9 editing sites are boxed. B, A model for the function of the A-U element. Editing factors assemble on the A-U element of the pre-edited mRNA (Fig. 5) and transfer the editing factors to its A-U element.

complexes. The cytochrome b gRNA I competes 20–80-fold better than the ferritin competitor (Fig. 5) for binding to the proteins comprising complexes C, E, and F. Thus, changes to the gRNA templating sequence that disrupt interactions with
editing components would be predicted to be detrimental even if the potential to guide a functional coding sequence within the pre-edited mRNA was maintained. As a result, there could be two different evolutionary forces conserving this sequence and limiting the opportunity for genetic drift.

The intermediates produced during the gRNA-independent reaction are consistent with the endonuclease/U-addition/ligation mechanism previously proposed for gRNA-directed editing based on the following observations. First, there is a ligation activity within the fractionated mitochondrial extract that co-fractionates with the gRNA-independent U-insertion activity. Linear molecules containing 3'-OH and 5'-phosphate groups, the same groups produced during gRNA-directed editing (24, 25), are chased into products by the co-fractionating ligation activity whereas RNAs containing a 3'-phosphate and 5'-OH are not. Second, there is significant accumulation of linear RNAs corresponding to cleavage of the circular cytochrome b RNA at sites of U-insertion when the ligation activity is inhibited through the exclusion of NTPs from the reaction. Third, in the presence of UTP, U nucleotides are added to the 3'-end of the linear molecules. These apparent similarities in mechanism are consistent with the possibility that the gRNA-independent reaction exploits some of the same editing components used during gRNA-directed editing.

The A-U element could, thus, be serving as an assembly point for the editing machinery on those pre-edited mRNAs for which it is especially important to be efficiently edited (Fig. 7B). The original model for gRNA-directed editing, in fact, postulated that editing components may interact with the pre-edited mRNA independent of gRNA (4). The pre-assembled editing factors would be able to insert U nucleotides within both the 5' and 3' adjacent sequence (Fig. 7B, top). This would explain the localization of the majority of the in vitro gRNA-independent U-insertions (Fig. 2C). U-insertion components could be transferred to the A-U element of the gRNA upon its binding to the pre-edited mRNA (Fig. 7B, middle). Cytochrome b gRNA I was previously shown to specifically inhibit the independent U-insertion reaction (9, 10) and this, in part, could be a result of the machinery being transferred from the A-U element of the mRNA to that of the gRNA. Accurate editing would occur after the transfer of editing factors to the gRNA (Fig. 7B, bottom). Additional experiments are being performed to further test this model.

Although the A-U element has only been identified within cytochrome b gRNA I, there are several other kinetoplastid gRNAs that are very A-U rich. Selection-amplification (33, 34) is being used to identify the sequence permutations of the A-U element that are able to support the internal U-insertions. Knowing the permissible permutations will be informative in determining whether related elements are found on other gRNAs. It is also possible that there are gRNA and/or mRNA-specific factors required for editing. The latter possibility is likely since there are life cycle stages in T. brucei during which specific mRNAs are not edited, even though both the pre-edited mRNA and appropriate gRNA are present (35–37).

Although the A-U element is able to support the U-insertion reaction and form specific complexes with the mitochondrial extract, the reaction is significantly enhanced by the addition of 5'-nucleotides either as a result of the intramolecular ligation or an artificial extension (Fig. 4B). This additional requirement for efficient U-insertion could also limit inappropriate U-insertions by the editing components assembled on the mRNA in vivo. The enhancement could be occurring in vivo, however, if the cytochrome b pre-edited mRNA, like the mitochondrial genome of some eucaryotes (see Ref. 38; reviewed in Ref. 39) is transcribed as part of a polycistrionic precursor. Alternatively, an enhancement could possibly result from interactions between the 5' and 3'-ends of the mRNA during processing and translation (40, 41). There are examples of in vivo misediting of cytochrome b mRNA (42), and it is possible that some of these events are a consequence of having editing components assembled on the pre-edited mRNA independent of gRNA.

The enhancement of the gRNA-independent U-insertions by an artificial 5'-extension on the cytochrome b mRNA is in agreement with previous results from a primer extension-based assay (10). However, the results from the primer extension assay also suggested that the 5'-extension was required to be base paired, and no such requirement was detected in this study. The discrepancy probably is a result of the primer extension assay being limited to the detection of U-insertions within the first 2 editing sites. Subtle changes in the structure of the RNA could be influencing the sites at which the U-insertions are occurring. Although the A-U element is a major determinant of the reaction, we cannot eliminate the possibility that the reaction is also influenced by other sequence and/or structural elements. Changes in structure may account for the increased level of U-insertion observed with the deletion of the pre-edited region (Fig. 3B, lane 4).

A-U rich sequences are found within the 5'-untranslated region of several mitochondrial mRNAs. The L. tarentolae A-U element is not completely conserved within the cytochrome b mRNA of T. brucei and Crithidia fasciculata, but both of these related genera of trypanosomes have similar sequences immediately upstream of the editing sites (43). An A-U-rich sequence present within the 5'-untranslated region of the Saccharomyces cerevisiae cytochrome b mRNA has been implicated in an interaction with the translation machinery (44). This raises the intriguing possibility that the editing components interacting with the L. tarentolae A-U element could either be associated with the translation machinery or perhaps evolutionarily related.

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REFERENCES
1. Simpson, L., and Shaw, J. (1989) Cell 57, 355–366
2. Sloop, P., and Benne, R. (1997) Trends Microbiol. 5, 189–195
3. Stuart, K., Allen, T. E., Heidmann, S., and Seiwert, S. D. (1997) Microbiology. Mol. Biol. Rev. 61, 105–120
4. Blum, B., Bakalarana, N., and Simpson, L. (1990) Cell 60, 189–198
5. Seiwert, S. D., Heidmann, S., and Stuart, K. (1996) Cell 84, 831–841
6. Kable, M. L., Seiwert, S. D., Heidmann, S., and Stuart, K. (1996) Science 273, 1189–1195
7. Cruz-Reyes, J., and Sollner-Webb, B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8901–8906
8. Byrne, K., Connell, G. J., and Simpson, L. (1996) EMBO J. 15, 6758–6765
9. Frech, G. C., Bakalarana, N., Simpson, L., and Simpson, A. M. (1995) EMBO J. 14, 178–87
10. Connell, G. J., Byrne, E., and Simpson, L. (1997) J. Biol. Chem. 272, 4212–4218
11. Brady, P., Simpson, L., and Kretzer, F. (1974) J. Protozool. 21, 782–790
12. Milligan, J. F., and Uhlenbeck, O. C. (1989) Methods Enzymol. 180, 51–62
13. Konarzewa, M. M., and Sharp, P. A. (1986) Cell 46, 845–855
14. Sugisaki, H., and Takanami, M. (1993) J. Biochem. 268, 875–891
15. Puttaraju, M., and Been, M. D. (1995) Nucleic Acids Symp. Ser. 33, 49–51
16. Schindewolf, C. A., and Domdey, H. (1995) Nucleic Acids Res. 23, 1133–1139
17. Bohjianen, P. R., Colvin, R. A., Puttaraju, M., Been, M. D., and Garcia-Blanco, M. A. (1996) Nucleic Acids Res. 24, 3733–3838
18. Pollard, V. W., Harris, M. E., and Hajduk, S. L. (1992) EMBO J. 11, 4429–4448
19. Goringir, H. U., Kosalowski, D. J., Morales, T. H., and Stuart, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1776–1780
20. Read, L. K., Goringir, H. U., and Stuart, K. (1994) Mol. Cell. Biol. 14, 2629–2639
21. Peris, M., Frech, G. C., Simpson, A. M., Bringaud, F., Byrne, E., Bakker, A., and Simpson, L. (1994) EMBO J. 13, 1664–1672
22. Pan, T., and Uhlenbeck, O. C. (1995) Gene (Amst.) 125, 111–114
23. Blum, B., and Simpson, L. (1990) Cell 62, 391–397
24. Seiwert, S. D., Heidmann, S., and Stuart, K. (1996) Cell 84, 831–841
25. Kable, M. L., Seiwert, S. D., Heidmann, S., and Stuart, K. (1996) Science 273, 8901–8906
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1189–1195

26. Greer, C. L., Peebles, C. L., Gegenheimer, P., and Abelson, J. (1983) Cell 32, 537–546
27. White, T. C., and Borst, P. (1987) Nucleic Acids Res. 15, 3275–3290
28. Huang, J., and Van der Ploeg, L. H. (1988) Nucleic Acids Res. 16, 9737–9759
29. Bakalar, N., Simpson, A. M., and Simpson, L. (1989) J. Biol. Chem. 264, 18679–18686
30. Rusche, L. N., Cruz-Reyes, J., Piller, K. J., and Sollner-Webb, B. (1997) EMBO J. 16, 4069–4081
31. Zuker, M. (1989) Science 244, 48–52
32. Schmid, B., Riley, G. R., Stuart, K., and Goringer, H. U. (1995) Nucleic Acids Res. 23, 3095–3102
33. Tuerk, C., and Gold, L. (1990) Science 249, 505–510
34. Ellington, A. D., and Szostak, J. W. (1990) Nature 346, 818–822
35. Feagin, J. E., Jasmer, D. P., and Stuart, K. (1987) Cell 49, 337–345
36. Koslowsky, D. J., Riley, G. R., Feagin, J. E., and Stuart, K. (1992) Mol. Cell. Biol. 12, 2043–2049
37. Riley, G. R., Myler, P. J., and Stuart, K. (1995) Nucleic Acids Res. 23, 708–712
38. Ojala, D., Montoya, J., and Attardi, G. (1981) Nature 290, 470–474
39. Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333
40. Preiss, T., and Hentze, M. W. (1998) Nature 392, 516–520
41. Tarun, S. Z., Jr., and Sachs, A. B. (1996) EMBO J. 15, 7168–7177
42. Sturm, N. R., and Simpson, L. (1990) Cell 61, 871–878
43. Feagin, J. E., Shaw, J. M., Simpson, L., and Stuart, K. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 539–543
44. Mittelmeier, T. M., and Dieckmann, C. L. (1995) Mol. Cell. Biol. 15, 780–789