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Genetics

Formation of guide RNA/messenger RNA chimeric molecules *in vitro*, the initial step of RNA editing, is dependent on an anchor sequence

(transesterification/*Leishmania tarentolae*)

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ABSTRACT **Synthetic pre-edited messenger RNA (mRNA) and guide RNA (gRNA) for the 5′-edited maxicircle-encoded ND7 cryptogene from *Leishmania tarentolae* formed chimeric molecules upon incubation in the presence of a mitochondrial extract. These chimeric molecules consisted of the gRNAs covalently linked to the mRNAs by short oligo(U) tails at normal editing sites in most cases. Unlike the previously reported chimeras present in steady-state kinetoplast RNA, the *in vitro*-synthesized chimeras showed no editing of downstream editing sites. The synthesis of chimeric RNAs required ATP and was dependent on the formation of a gRNA/mRNA anchor duplex 3′ of the pre-edited region, as shown by *in vitro* mutagenesis of the mRNA and the gRNA. mRNA sequences 3′ and 5′ of the pre-edited region also affected the efficiency of the chimera-forming activity. This *in vitro* system may accurately represent the initial step in RNA editing.**

RNA editing in mitochondria of kinetoplastid protozoa is a posttranscriptional process resulting in the insertion and deletion of uridine (U) residues from maxicircle-encoded mRNA transcripts at defined sites, thereby creating an open reading frame and making the mRNAs functional for translation (1, 2). Small guide RNA (gRNA) transcripts from both the maxicircle and the minicircle DNA contain the sequence information for the edited regions and have been proposed to mediate this process (3–7). In the transesterification model of RNA editing (4, 8), U residues are directly transferred from the oligo(U) 3′ tail of the gRNA into the mRNA via a gRNA/mRNA chimeric intermediate. Such chimeric molecules have been found in steady-state mitochondrial RNA from *Leishmania tarentolae* (4) and *Trypanosoma brucei* (6). In *L. tarentolae*, these *in vivo* chimeric molecules generally consist of gRNA covalently linked via the 3′ oligo(U) sequence to the corresponding mRNA at a normal editing site, with the downstream editing sites being fully edited (4).

Two recent reports have shown that gRNA/mRNA chimeric molecules can be formed *in vitro* in the presence of mitochondrial extracts from *T. brucei*. Harris and Hajduk (9) used labeled synthetic gRNA or mRNA to monitor the formation of chimeric molecules for the cytochrome *b* cryptogene by gel electrophoresis. Koslowsky *et al.* (10) used PCR amplification to analyze gRNA/mRNA chimeric products obtained with A6 (= MURF4) pre-edited mRNA and gA6-14 gRNA.

In this study we report an *in vitro* system for chimeric formation of synthetic gND7-II gRNA and synthetic pre-edited ND7 mRNA from *L. tarentolae*. The products of this reaction were analyzed by cloning and sequencing and shown to be similar but not identical to *in vivo* chimeric molecules, and *in vitro* mutagenesis was performed to establish the role of the "anchor" sequence in this initial step of RNA editing.

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MATERIALS AND METHODS

Growth of Cells and Preparation of Mitochondrial Extracts. *L. tarentolae* cells (UC strain) were grown as described (11). The kinetoplast–mitochondrial fraction was isolated from cells in midlogarithmic growth by Renografin density gradient flotation (12, 13). The mitochondrial fraction from 1 liter of culture was disrupted by sonication (Braunsonic 1510, 100 W, microtip, three 20-sec periods, 5°C) after swelling for 10 min in 2 ml of 10 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$. For low-salt extraction, an equal volume of 2× extraction buffer [40 mM Hepes, pH 7.9/40% (vol/vol) glycerol/0.2 M KCl/0.4 mM EDTA/1 mM phenylmethanesulfonyl fluoride/1 mM dithiothreitol] was added, and the solution was clarified by centrifugation at 50,000 × *g* for 30 min. For high-salt extraction, 2 ml of 2× high-salt extraction buffer [40 mM Hepes, pH 7.9/50% (vol/vol) glycerol/0.84 M NaCl/0.4 mM EDTA/1 mM phenylmethanesulfonyl fluoride/1 mM dithiothreitol] was added after sonication. The extract was gently agitated with a magnetic stirrer for 30 min on ice prior to centrifugation at 50,000 × *g* for 30 min. The extract was then dialyzed against 1× extraction buffer at 4°C overnight by using centrifugal microconcentrators (Centricon 10, Amicon). Any insoluble precipitate formed during dialysis was pelleted by centrifugation. The supernatant was divided into 50-μl aliquots and frozen in dry ice/ethanol. The extracts, stored at −80°C, maintained chimera-forming activity for up to 3 months.

mRNA and gRNA Substrates. The RNA substrates were made by T7 polymerase transcription from DNA templates obtained by PCR. The truncated substrate mRNAs contained only the 5′ editing domain together with the *in vivo* 5′ terminus and some 3′ flanking sequence.

The ND7 gRNA was transcribed from PCR-amplified DNA in the presence of [α-^{32}P]UTP. The DNA template encoded 13 U residues at the 3′ end. The products of T7 transcription exhibited some heterogeneity at the 3′ end, due to both premature termination during the *in vitro* transcription and *in vitro* terminal nucleotidyltransferase activity. The gRNA transcripts with 8–13 U residues at the 3′ end were purified by polyacrylamide gel electrophoresis.

Fig. 1 shows a compilation of the RNA substrates used in this study.

***In Vitro* Chimera Formation Assay.** T7-transcribed mRNA and uniformly ^{32}P-labeled gRNA were mixed and briefly denatured at 70°C prior to annealing at 37°C and 25°C for 10 min each, in 2.5 μl of 20 mM Hepes, pH 7.9/100 mM KCl/1 mM EDTA. Eight microliters of reaction buffer [8% (wt/vol) PEG 8000/12.5 mM MgCl/2.5 mM ATP containing RNasin (Promega; 1 unit/μl)] and 15 μl of thawed mitochondrial

Abbreviations: gRNA, guide RNA; nt, nucleotide(s).

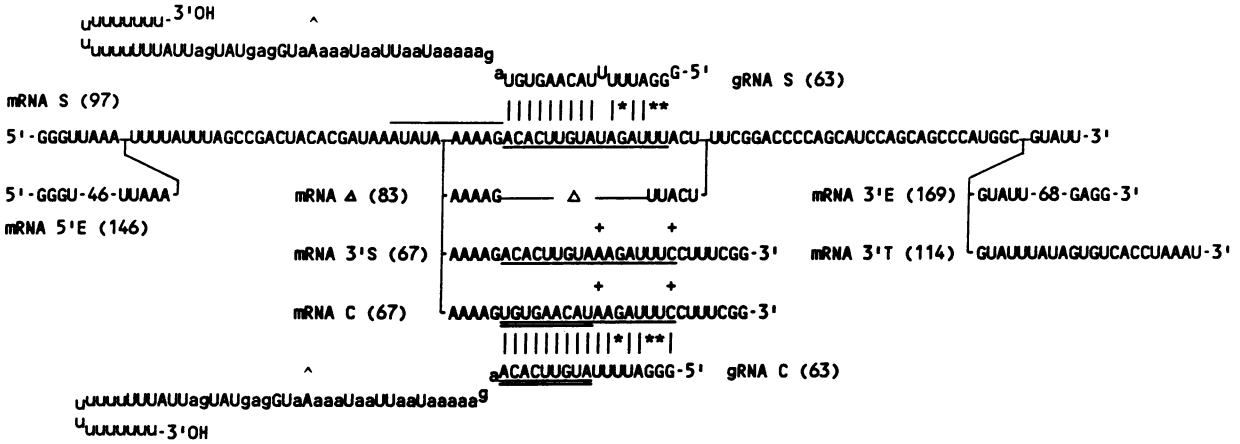

FIG. 1. Synthetic mRNA and gRNA molecules used for *in vitro* chimera formation. The length of each construct in nucleotides is shown in parentheses next to its designation. The pre-edited region of mRNA S is indicated by an overline and the anchor sequence is indicated by an underline. The compensatory changes introduced into mRNA C and gRNA C are shown by double underlining. Note that the 3′ ends of the gRNAs each contain 13 U residues. The gRNA and mRNA transcripts were purified by gel electrophoresis after transcription by T7 polymerase from DNA templates obtained by PCR amplification of kinetoplast DNA. The gRNAs were transcribed in the presence of [α-^{32}P]UTP, which labels the entire molecule but in particular the 3′ oligo(U) tail, which is in this case encoded by the DNA template. A single-site U → A mutation present in the substrate gRNAs is indicated by a caret. This mutation allows for distinction between exogenous and endogenous gRNA sequences. Two single-site mutations marked with plus signs have been introduced into mRNA 3′S and mRNA C for the same purpose.

extract were added and incubated at 27°C for up to 2 hr. The reaction was stopped with 100 μl of proteinase K solution [0.25% (wt/vol) *N*-lauroylsarcosine/25 mM EDTA containing proteinase K (0.25 unit/ml)] at 37°C for 20 min. RNA was recovered by phenol extraction and ethanol precipitation. The resulting RNA products were electrophoresed in denaturing polyacrylamide/urea gels and detected by autoradiography.

Sequencing of *in Vitro* Chimeric RNAs. The RNA products of an *in vitro* reaction (see Fig. 2*C*, lane 6) were purified by gel electrophoresis and amplified by PCR using a 3′ primer for reverse transcription which is selective for the 3′ tag sequence on mRNA 3′T (Fig. 1) and a 5′ primer which is specific for the gRNA. Cloning and sequencing were performed as described (4).

Oligodeoxynucleotides. The following oligodeoxynucleotides were synthesized on an Applied Biosystems 381A synthesizer and were purified by thin-layer chromatography:

5′-TAATACGACTCACTATAGGGTTATGGTAATTAGTTACAGT-3′ (5′ primer, mRNA 5′E);

5′-CCTCGATGTAAATACCCAATAATTAC-3′ (3′ primer, mRNA 3′E);

5′-AATACGCCATGGGCTGCTGGATG-3′ (3′ primer, mRNA S/5′E/Δ/3′S/C);

5′-TAATACGACTCACTATAGGGATTTTTACAAGTGTAGAAAA-3′ (5′ primer, gRNA S);

5′-AAAAAAAAAAAAATAATCATACTCCATTT-3′ (3′ primer, gRNA S);

5′-TAATACGACTCACTATAGGGTTAAATTTTATTTAGCCGAC-3′ (5′ primer, mRNA S/3′T/3′E/3′S/Δ/C);

5′-ATTTTTACAAGTGTAGAAAA-3′ (5′ primer, gRNA S, PCR analysis);

5′-ATTTAGGTGACACTATAAATACGCCATGGGCTGCTGG-3′ (3′ primer, mRNA 3′T);

5′-ATTTAGGTGACACTATAAAT-3′ (3′ primer, mRNA 3′T, PCR analysis);

5′-CTACACGATAAATATAAAAAGTTACTTTCGGACCCCAGC-3′ (5′ primer, mRNA internal, creates deletion);

5′-GCTGGGGTCCGAAAGTAACTTTTTATATTTATCGTGTAG-3′ (3′ primer, mRNA internal, creates deletion);

5′-CCGAAAGGAAATCTTATGTTCACACTTTTTATATTTATCG-3′ (3′ primer, mRNA 3′S);

5′-CCGAAAGGAAATCTTTACAAGTGTCTTTTTA-3′ (3′ primer, mRNA C);

5′-TAATACGACTCACTATAGGGATTTTATGTTCACAAGAAAAATAATTAATAAATATGGAG-3′ (5′ primer, gRNA C).

RESULTS

Synthetic ND7 gRNA and mRNA Constructs. Editing of the 5′ end of the ND7 mRNA in *L. tarentolae* is mediated by a single gRNA (gND7-II) and consists of the addition of 20 U residues at seven sites (2, 3). The 3′ gRNA/mRNA anchor duplex contains a total of 13 base pairs with a single mismatch occurring after a stretch of nine consecutive base pairs. To analyze the interaction of the ND7 gRNA and the ND7 mRNA *in vitro*, several constructs were synthesized by PCR amplification and T7 transcription as shown in Fig. 1. The synthetic gRNA (gRNA S) contained 8–13 nonencoded U residues at the 3′ end, since that is close to the average number of U residues for *in vivo* gRNAs. The gRNA also contained three nonencoded G residues at the 5′ end which are required for optimal T7 transcription, but these nucleotides actually increased the stability of the anchor duplex. A single U → A mutation (Fig. 1, ^) was incorporated into gRNA S to distinguish between exogeneous and endogeneous gRNA sequences.

The synthetic pre-edited ND7 mRNA S construct consisted of the 10-nucleotide (nt) pre-edited region and the 16-nt anchor sequence together with 43 nt of 5′ flanking and 38 nt of 3′ flanking sequence. The 5′ end of the mRNA S construct corresponded to the 5′ end of the mRNA, as determined previously by primer extension, together with three G residues added to optimize T7 transcription.

In vitro mutagenesis of the gRNA and the mRNA was performed by recombinant PCR followed by T7 transcription. The constructs consisted of 5′- or 3′-extended mRNAs, anchor-deleted mRNA, anchor-substituted mRNA, and anchor-substituted gRNA. These are described in Fig. 1.

Transfer of Label from ND7 gRNA to a High Molecular Weight Band Is Dependent on ND7 mRNA and Mitochondrial Extract. ND7 gRNA was transcribed using [^{32}P]UTP. This effectively labels the 3′ oligo(U) tail in addition to some internal U residues. The U-labeled synthetic ND7 gRNA (gRNA S in Fig. 1) and unlabeled synthetic ND7 mRNA (mRNA S in Fig. 1) were annealed and incubated in the presence of a soluble mitochondrial extract obtained by sonication and salt extraction. A transfer of ≈5% of the label from the gRNA to two high molecular weight bands was observed (Fig. 2*A*, lane 3). No transfer occurred in the absence of extract or mRNA.

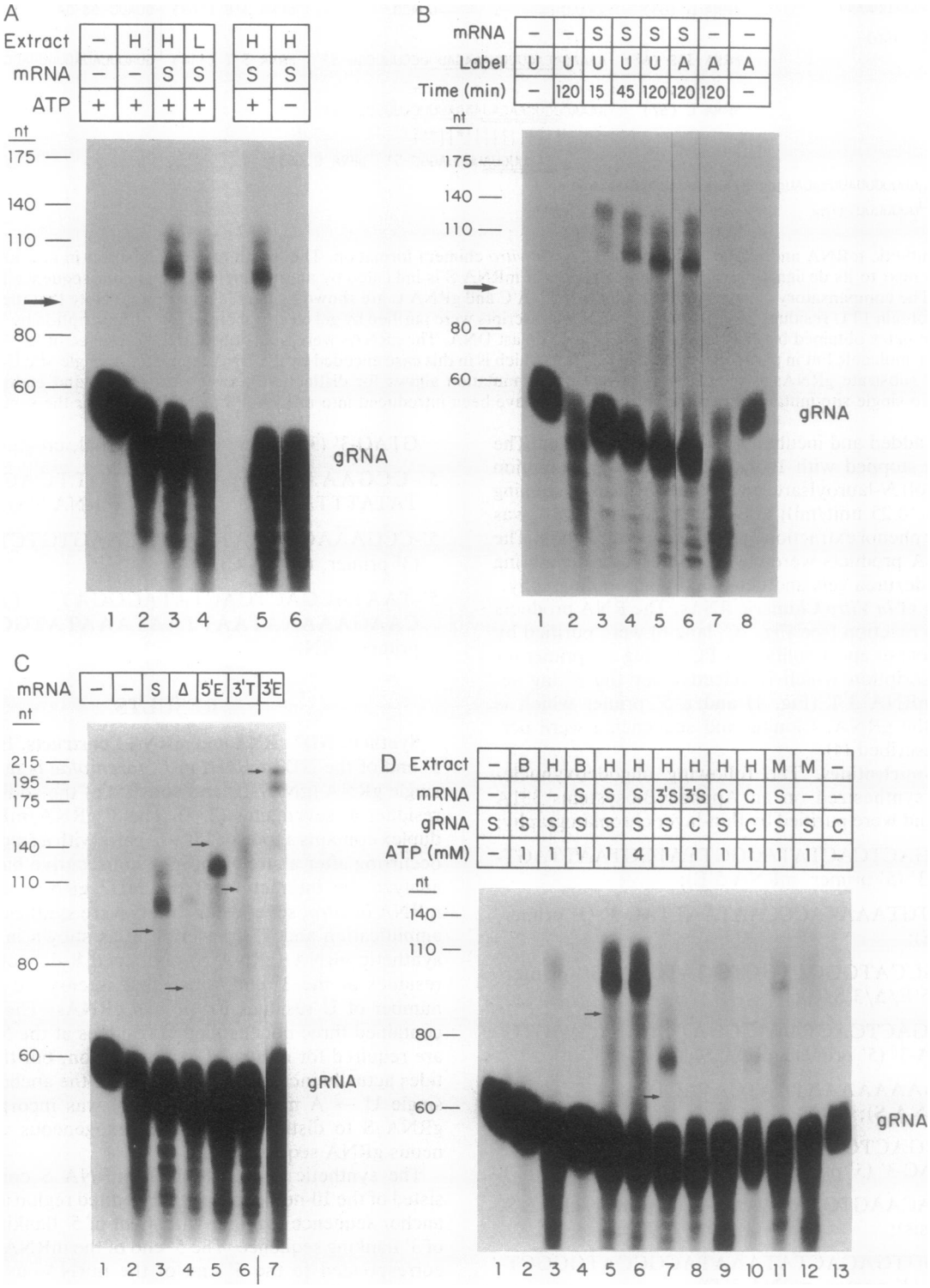

FIG. 2. Polyacrylamide gel analysis of *in vitro* formation of gRNA/mRNA chimeras. The identity of the mRNA and gRNA, the type of extract, and the type of labeled nucleotide are indicated above the lanes. (*A*) Transfer of label from [^{32}P]UTP-labeled gRNA S to mRNA S. In lanes 1–5, 1 mM ATP was added. H, high-salt mitochondrial extract; L, low-salt mitochondrial extract. Arrow indicates the size of the input mRNA. (*B*) Time course of transfer of label from [^{32}P]UTP-labeled gRNA S to mRNA S, and effect of labeling the gRNA with [^{32}P]ATP. U, [^{32}P]UTP-labeled gRNA; A, [^{32}P]ATP-labeled gRNA. (*C*) Effect of anchor deletion and 3′ and 5′ extensions of the mRNA. Arrows indicate the sizes of the added mRNAs. (*D*) Compensatory base changes restore chimera formation. M, supernatant from frozen–thawed mitochondria; B, extract buffer alone. In lane 6, 4 mM ATP was added stepwise during the incubation period.

The transfer reaction required ATP (Fig. 2*A*, lanes 5 and 6). The ATP in the standard reaction was not rate-limiting, since stepwise addition of 4 mM ATP during the 2-hr incubation had no significant stimulatory effect over the prior addition of 1 mM ATP (Fig. 2*D*, lanes 5 and 6). A low-salt extraction of sonicated mitochondria also yielded high molecular weight material, but the products were slightly less discrete (Fig. 2*A*, lane 4). The supernatant from unextracted frozen–thawed mitochondria also showed some transfer activity (Fig. 2*D*, lane 11). Extracts prepared by 0.3% Triton X-100 lysis of the mitochondria were inactive (data not shown).

Identification of the Transfer Products as Chimeric Molecules. The size of the transfer products as indicated from the gel mobility would be consistent with either partially edited mRNA or gRNA/mRNA chimeric molecules (4). The chimeric nature of these products was demonstrated by performing a transfer experiment with [^{32}P]ATP-labeled gRNA. Since A residues are internal in the gRNA, any transfer of label should be the result of chimera formation. The products of this reaction were identical to those obtained with [^{32}P]UTP-labeled gRNA (Fig. 2*B*, lanes 5 and 6).

This conclusion was strengthened by experiments in which target mRNAs of different sizes were used (Fig. 2*C*, lanes 5–7). A 5′-extended mRNA (mRNA 5′E in Fig. 1; Fig. 2*C*, lane 5) yielded a single upper band comigrating with the minor band obtained by using the standard mRNA S (lane 3). Two 3′-extended mRNAs (mRNAs 3′T and 3′E in Fig. 1; Fig. 2*C*, lanes 6 and 7) yielded high molecular weight bands larger than the bands formed with the standard mRNA, as expected for chimeric molecules. These latter products were in lower yield than the products obtained with the standard mRNA, suggesting that the 3′ extensions somehow interfered with chimeric formation. Use of a 3′-truncated mRNA (mRNA 3′S in Fig. 1; Fig. 2*D*, lane 7) also yielded the expected shortened product, again with somewhat lower efficiency.

A time course of the reaction with the standard mRNA S (Fig. 2*B*, lanes 3–5) showed that label first appeared in the upper band in 15 min and then in the lower band after 2 hr of incubation, suggesting a kinetic relationship. We speculate that the lower band represents a more stable chimeric molecule, but the exact nature of the differences between these two bands is unknown.

PCR Amplification and Sequencing of Transfer Products. Definitive evidence for the generation of chimeric molecules in the *in vitro* reaction was obtained by selective PCR amplification of transfer products obtained by using the 3′-tagged mRNA 3′T (Fig. 2*C*, lane 6). The 3′ tag sequence, which was unique to the exogeneous mRNA, was used for reverse transcription to make cDNA and as a 3′ primer sequence for PCR, and an internal gRNA sequence was used for the 5′ primer for PCR (12). The PCR products were cloned and 15 random positive clones were sequenced. Fourteen of the clones showed gRNAs attached at editing sites 2, 3, 4, 5, and 7 (Fig. 3). A single clone showed a gRNA attached 4 bases upstream of the pre-edited region. The presence of the single U → A base mutation in the gRNA (Fig. 1) introduced into the synthetic gRNA during the synthesis showed that the

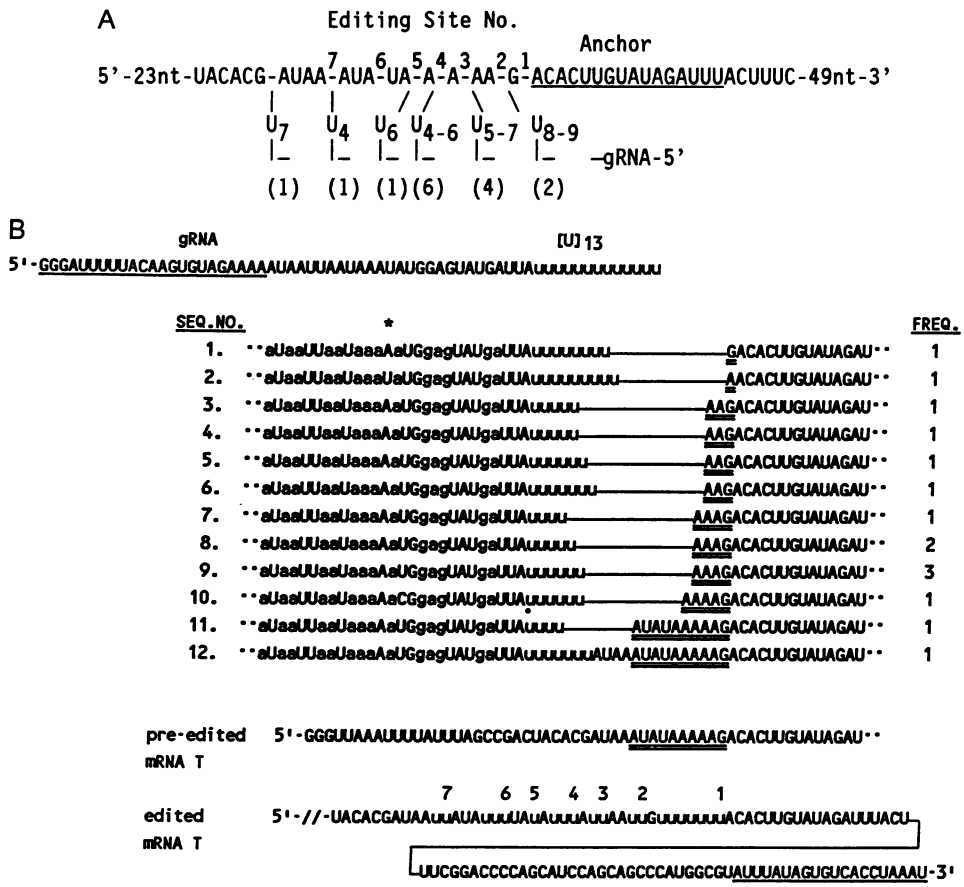

FIG. 3. Sequences of gRNA/mRNA chimeras obtained by PCR amplification of the gel-isolated smear located above the position of the input mRNA, 3′T, produced after incubation of gRNA S and mRNA 3′T with mitochondrial extract. (*A*) Sequence of the pre-edited ND7 mRNA, shown with the attachment sites of the gRNAs indicated schematically together with the number of U residues between the gRNA and the mRNA for each clone. The number of clones obtained with gRNAs attached at the site indicated is shown in parentheses. (*B*) Actual sequences of the 15 clones obtained from PCR-amplified DNA. The original gRNA sequence is shown at the top, and the edited and pre-edited mRNA sequences are shown at the bottom. The pre-edited sequence is indicated by double underlines. The single U → A mutation introduced into the gRNA is indicated by a star. FREQ., no. of clones.

exogeneous gRNA was utilized for 14 of the 15 chimeric molecules. The chimeras had 4–9 U residues linking the gRNA and the mRNA, presumably derived from the original 8- to 13-mer oligo(U) tails, which were probably shortened as a result of exonuclease activity in the extract (see Fig. 2*A*, lane 2). Differences from the previously reported *in vivo* chimeras (4) include the absence of chimeras with gRNAs attached at editing site 1 and the complete lack of editing of any sites downstream of the attachment sites.

Anchor Dependence of Chimera Formation. The mRNA 3′ anchor sequence, which is located just downstream of the pre-edited region, has been proposed to represent a target for the initiation of editing by formation of a base-paired duplex with the 5′ end of the corresponding gRNA (3).

An anchor requirement for the *in vitro* formation of chimeric molecules was shown by use of an mRNA which had the anchor sequence deleted (mRNA Δ in Fig. 1). This deletion resulted in a complete inhibition of the formation of the expected band (Fig. 2*C*, lanes 3 and 4). However, a minor transfer of label occurred to a band that was larger than expected. This could have been due to the formation of a secondary anchor.

An inhibition of the transfer of label to an upper band was also observed (Fig. 2*D*, lane 9) by using an *in vitro*-mutated mRNA (mRNA C in Fig. 1) that contained a substituted anchor sequence which could not base pair with the standard gRNA (gRNA S in Fig. 1). Likewise, use of the 3′-truncated mRNA with the native anchor sequence (mRNA 3′S in Fig. 1) together with an anchor-substituted gRNA (gRNA C in Fig. 1) showed no transfer of label (Fig. 2*D*, lane 8). However, use of the mutated mRNA C together with the gRNA C, which contained compensatory base changes to allow the formation of an anchor duplex, restored the transfer of label (lane 10). The apparent lower efficiency of transfer in this experiment was mainly a result of a lower specific activity of this particular gRNA. The minor high molecular weight band observed in lane 9 was also present in the no-mRNA control in lane 3 and therefore represents a transfer of label to some endogeneous RNA. We conclude that the presence of a gRNA/mRNA anchor duplex is required for chimera formation *in vitro*.

DISCUSSION

We have developed an *in vitro* system from *L. tarentolae* mitochondria which exhibits gRNA/mRNA chimera formation, and we have shown that this activity is dependent on the presence of an anchor sequence that allows hybridization of the 5′ end of the gRNA and the mRNA. The formation of chimeric molecules was demonstrated directly by following a transfer of label from U-labeled gND7-I gRNA to a high molecular weight band and indirectly by sequence analysis of PCR-amplified transfer products.

The formation of chimeras was entirely dependent on the addition of mitochondrial extract and required ATP. Extracts prepared from sonicated mitochondria by high-salt elution yielded the best results. We have found recently that the chimera-forming activity sediments in glycerol gradients as a 15–25S peak, depending on the preparation (B.B., A. Bakker, and L.S., unpublished results), suggesting the participation of a high molecular weight complex.

A similar chimera-forming activity from mitochondria of *T. brucei* has been reported (9, 10). In the Harris and Hajduk study (9), synthetic pre-edited CYb mRNA was used together with a synthetic gRNA covering the first three editing sites. The sequence of the gRNA was taken from the *L. tarentolae* gCYb-I gRNA, since no CYb gRNAs have yet been identified for *T. brucei*. The choice of a heterologous gRNA sequence, however, may not accurately simulate the *in vivo* situation. Harris and Hadjuk (9) were unable to PCR amplify the *in vitro* products and therefore attempted to directly sequence 5′-end-labeled chimeric molecules. Only very limited direct sequence information was obtained from the *in vitro* products, and the site of attachment of the gRNA was therefore ambiguous.

Koslowsky *et al.* (10) employed PCR amplification to analyze chimeric molecules formed with synthetic A6 (= MURF4) pre-edited mRNA and synthetic gA6-14 gRNA. However, the amplified *in vitro* chimeric molecules were unusual in that they lacked a stretch of U residues linking the gRNA to the mRNA and most of the gRNAs were truncated at variable sites at the 3′ end, suggesting that the process was aberrant in some respect. In both studies, specificity was demonstrated by showing that a variety of heterologous RNAs did not form chimeric molecules with added gRNA. In both studies (9, 10) a requirement for a gRNA 3′-terminal hydroxyl group was demonstrated and addition of protease-sensitive mitochondrial extract was required for chimera formation.

The chimeric molecules formed in our *in vitro* system are similar but distinct from those found in steady-state kinetoplast RNA *in vivo*. They are similar in that gRNA attachment generally occurs at normal mRNA editing sites. The *in vivo* chimeras, however, have the downstream sites fully and correctly edited (4), whereas the *in vitro* chimeras exhibit no editing at any site. One possible explanation is a failure to rejoin the two separated mRNA fragments after chimeric formation. Alternatively, correctly edited products may actually be formed during the incubation but may undergo rapid hydrolysis (14). This would be consistent with the observed lack of chimeric molecules with gRNAs attached at editing site 1, which is the preferred attachment site for *in vivo* chimeric molecules (4). The remaining stable chimeric molecules observed in this study would then represent aberrant products accumulating during the incubation. Nevertheless, the observed *in vitro* formation of chimeric RNAs may still accurately reflect the initial step of RNA editing, consistent with the transesterification model for RNA editing (4, 8). Alternatively, chimeric molecules could be created by site-specific cleavage (14) and adventitious ligation of the 3′ oligo(U) tail of the gRNA to the cleaved mRNA 3′ fragment, in line with the enzyme cascade model (3). The development of an accurate and complete *in vitro* editing system will be required to definitively distinguish between these models, but the availability of an *in vitro* system for the formation of chimeric gRNA/mRNA molecules should allow a precise dissection of the sequence requirements for chimera formation and also a fractionation of the extract components required for this activity.

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