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# Internal frameshifts within the mitochondrial genes for cytochrome oxidase subunit II and maxicircle unidentified reading frame 3 of *Leishmania tarentolae* are corrected by RNA editing: Evidence for translation of the edited cytochrome oxidase subunit II mRNA

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ABSTRACT **The *Leishmania tarentolae* cytochrome oxidase (EC 1.9.3.1) subunit II (COII) and maxicircle unidentified reading frame 3 (MURF3) mRNAs are edited internally by the addition of four and five uridine residues, respectively, which eliminate −1 and +1 reading frameshifts in the gene sequences. The editing events in COII are conserved in three kinetoplastid species, and those in MURF3 are conserved in two species. A primer extension assay showed that the ratio of edited to unedited RNA differed for each gene: 89% of the COII and 36% of the MURF3 transcripts are edited. Preliminary evidence was obtained for translation of the edited COII transcript into protein: antibodies generated against a synthetic peptide with the predicted carboxyl-terminal amino acid sequence reacted with a polypeptide of the correct molecular weight in immunoblots of a mitochondrial lysate.**

The nucleotide sequences of transcripts of several mitochondrial cryptogenes in kinetoplastid protozoa are modified by the process of RNA editing (1–6). Three kinetoplastid mitochondrial genes—cytochrome oxidase (EC 1.9.3.1) subunit II (COII) and maxicircle unidentified reading frames 2 and 3 (MURF2 and MURF3)—contain conserved internal frameshifts in their protein coding regions (7–11). The addition of four uridine residues at three sites within the *Trypanosoma brucei* and *Crithidia fasciculata* COII transcripts overcome a −1 frameshift (24), and the addition of five uridine residues at three sites within the *C. fasciculata* (12) MURF3 transcripts overcome a +1 frameshift. In this paper we examine the editing of the COII and MURF3 cryptogenes of *Leishmania tarentolae*.

## MATERIALS AND METHODS

**Cell Culture and Mitochondrial Isolation.** *L. tarentolae* (UC strain) cells were grown, and mitochondria were isolated as described (13–15).

**Nucleic Acid Isolation.** Mitochondrial kinetoplast RNA (kRNA) was isolated from mitochondrial pellets as described (15). The isolation procedure involves several DNase I digestions, which eliminate any kinetoplast DNA (kDNA) contamination. Poly(A)$^+$ kRNA was prepared by poly(U)-Sephadex chromatography. Network kDNA, *Eco*RI-linearized maxicircle DNA, and nuclear DNA were isolated as described (16).

**Preparation of Mitochondrial (Kinetoplast) Lysate.** Mitochondria were solubilized, and a cytochrome-enriched red-band fraction was isolated as described (17).

**RNA and DNA Sequencing.** Two to five micrograms of *L. tarentolae* poly(A)$^+$ kRNA or 2 μg of *L. tarentolae* maxicircle DNA was hybridized to 5′ end-labeled primers, and dideoxy chain-termination sequencing was performed using avian myeloblastosis virus reverse transcriptase (3).

**Primer Extension Analysis.** $^{32}$P-end-labeled primers were hybridized to total kRNA and extended by using avian myeloblastosis virus reverse transcriptase in the absence of specific dNTPs. A Bio-Rad densitometer was used to quantitate the relative peak areas.

**Oligonucleotides.** The following oligonucleotide primers were used in this study: S19, 5′-CGTATTGCTAGACAGCATC-3′ (nucleotides 3587–3569) (MURF3); S37, 5′-ACAGAGTTCACTACATTGTCC-3′ (nucleotides 10080–10060) (COII); S39, 5′-CCAGTCTATCAAAATACGGTAAACAC-3′ (nucleotides 3508–3488) (edited MURF3); S40, 5′-AAACTACACTTACATAATCCAG-3′ (nucleotides 3526–3505) (MURF3); S42, 5′-CCAGGTTCTCTACTTTAACTCCT-3′ (nucleotides 10009–9987) (COII); S43, 5′-CCAGGTATACAATCTACTTTAAC-3′ (nucleotides 10009–9991) (edited COII); S62, 5′-GAACTATTTCATTACAGCGACCAGG-3′ (nucleotides 10029–10005) (COII). Numbering begins from the single *Eco*RI site 5′ of the 12S RNA gene (7). Oligonucleotides were labeled by using phage T4 polynucleotide kinase (BRL) and [γ-$^{32}$P]dATP (ICN; >7000 Ci/mmol; 1 Ci = 37 GBq) as described (18).

**DNA Hybridization.** Restriction nuclease digestions were performed under conditions recommended by the suppliers (New England Biolabs and BRL). Agarose gels were blotted to Nytran filters (Schleicher & Schuell). Hybridization probes were either $^{32}$P-5′-end-labeled oligonucleotides ($10^9$ cpm/μg) or nick-translated cloned gene fragments ($10^8$ cpm/μg).

Oligonucleotide hybridizations were performed in 0.90 M NaCl/0.09 M sodium citrate, pH 7/0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.05% sodium pyrophosphate; 0.1% sodium dodecyl sulfate (SDS)/20 μg of yeast tRNA per ml at 37°C. Washes were performed in tetramethylammonium chloride (19) with the addition of 1% SDS. All oligonucleotide hybridizations described here were washed at 4°C below the melting temperature. Gene fragment hybridizations were carried out as described (18).

**Synthetic Peptide.** A peptide corresponding to the predicted C-terminal sequences of the *L. tarentolae* COII protein (Gly-Val-Leu-His-Gly-Phe-Met-Pro-Ile-Val-Ile) was synthesized by using an added lysine as the N-terminal residue for glutaraldehyde coupling. HPLC analysis performed on a μBondapak $C_{18}$ column (Waters) showed the peptide to be >95% pure. The peptide was coupled with glutaraldehyde to either bovine serum albumin or ovalbumin as described (20) except that the buffer was 0.13 M sodium borate (pH 9.0).

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Abbreviations: kRNA, kinetoplast RNA; kDNA, kinetoplast DNA; COII, cytochrome oxidase subunit II; MURF3, maxicircle unidentified reading frame 3.

The conjugate was dialyzed extensively against 10 mM Tris, pH 7.4/140 mM NaCl.

**Preparation of Antiserum and Immunological Procedures.** Female New Zealand White rabbits were injected subcutaneously at four to five sites at 2-week intervals with 0.25–0.55 mg of the peptide–bovine serum albumin conjugate (containing 50–100 $\mu$g of peptide) in either Freund's complete or incomplete adjuvant. Serum was monitored for antibody activity with an ELISA as described (21) by using the COII peptide–ovalbumin conjugate as the coating antigen in the microtiter wells. Analysis of antibodies by immunoblotting (Western analysis) was performed as described (22), except that 4-chloro-1-naphthol was used as the chromogen. Electrophoresis through 15% polyacrylamide/0.1% SDS/8 M urea slab gels was carried out as described (23). Preimmune serum did not react with the mitochondrial protein fraction on an identical blot (see Fig. 6*A*). The specificity of the antiserum for the native COII polypeptide was verified by preadsorption of the immune sera with 6 mM peptide prior to reaction with the Western blot and by a competitive ELISA assay using ovalbumin-conjugated peptide adsorbed in microtiter wells (data not shown).

## RESULTS

***L. tarentolae* COII and MURF3 Transcripts Are Edited by the Addition of Four and Five Uridine Residues in the Frameshift Region.** The COII transcripts are modified by the addition of four uridine residues that are absent from the DNA sequence (Fig. 1*A*). These uridine additions occur in the same relative positions as those described for the *T. brucei* and *C. fasciculata*-edited mRNAs and correct the −1 frameshift in the genomic sequence. The three amino acids created by editing in the *L. tarentolae* COII protein are identical to those in the *T. brucei* and *C. fasciculata* predicted sequences.

The two overlapping reading frames encoded by the MURF3 gene sequence are joined by the addition of five uridine residues in the transcript (Fig. 1*B*), in identical relative locations to those found in edited *C. fasciculata* MURF3 transcripts (12). The three new amino acids created by these uridine additions are also identical to those created by editing in *C. fasciculata*. Extension products (>) of unknown significance occur seven nucleotides downstream of the MURF3 editing sites and five nucleotides downstream of the COII editing sites.

**Templates for Edited COII and MURF3 Transcripts Are Not Present in the Mitochondrial or Nuclear DNA.** When oligonucleotide S43 (complementary to the edited COII sequence) or a 0.6-kilobase (kb) gene fragment were used as probes under conditions in which a single copy of the unedited gene could be detected by an unedited probe, no hybridization signals were obtained in either kDNA or nuclear DNA, indicating that edited versions of the COII gene sequence do not exist in mitochondrial or genomic DNA even as single copy sequences (Fig. 2). Similar negative hybridization results were obtained for the edited MURF3 gene when using the S39 edited oligomer probe (Fig. 3) and a 1.17-kb gene fragment containing the unedited sequence as a control.

**Different Relative Amounts of Edited and Unedited Transcripts Exist for the COII and MURF3 Sequences.** We have used a −dNTP primer extension assay to measure the relative amounts of edited and unedited species in the steady-state RNA population and to search for partially edited molecules. In Fig. 4*A* the S62 primer was hybridized to

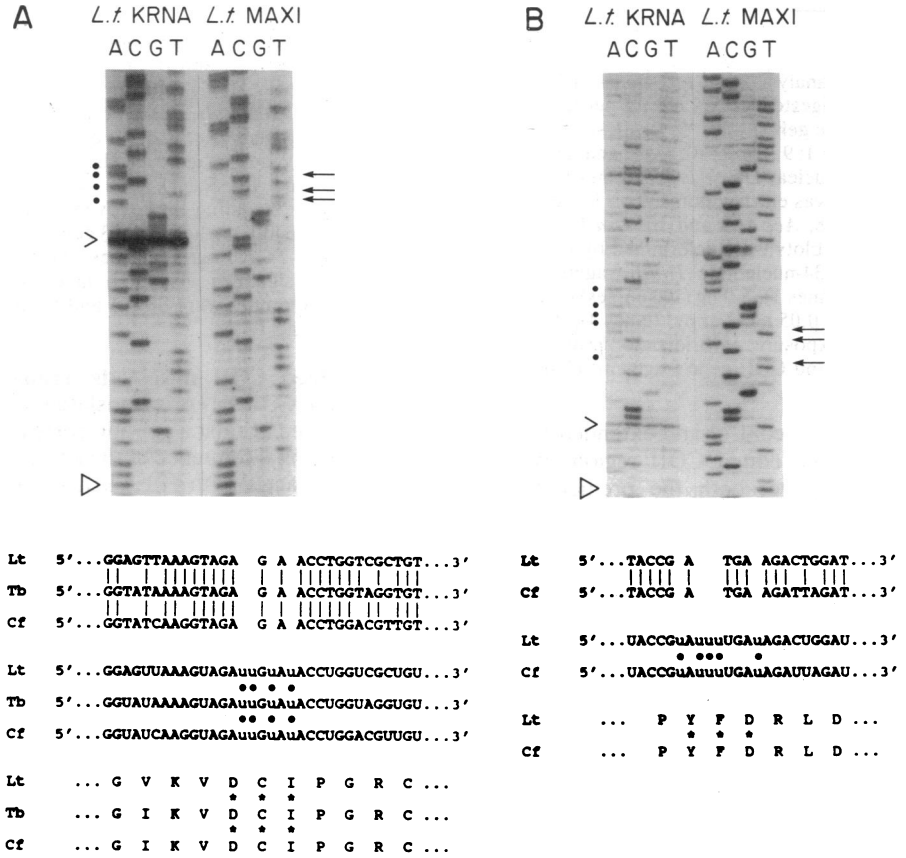


FIG. 1. Sequences of internal-edited COII (*A*) and MURF3 (*B*) mRNAs from *L. tarentolae* (*L.t.*). Comparisons of the sequences for three kinetoplastid species (12, 24) are shown below the gels. The open arrowheads mark equivalent locations in the RNA and DNA ladders 3′ of the editing events, the dots represent added uridine residues, and the arrows indicate the location of the added uridine residues in the DNA ladder. Primers used were S37 for COII and S19 for MURF3.

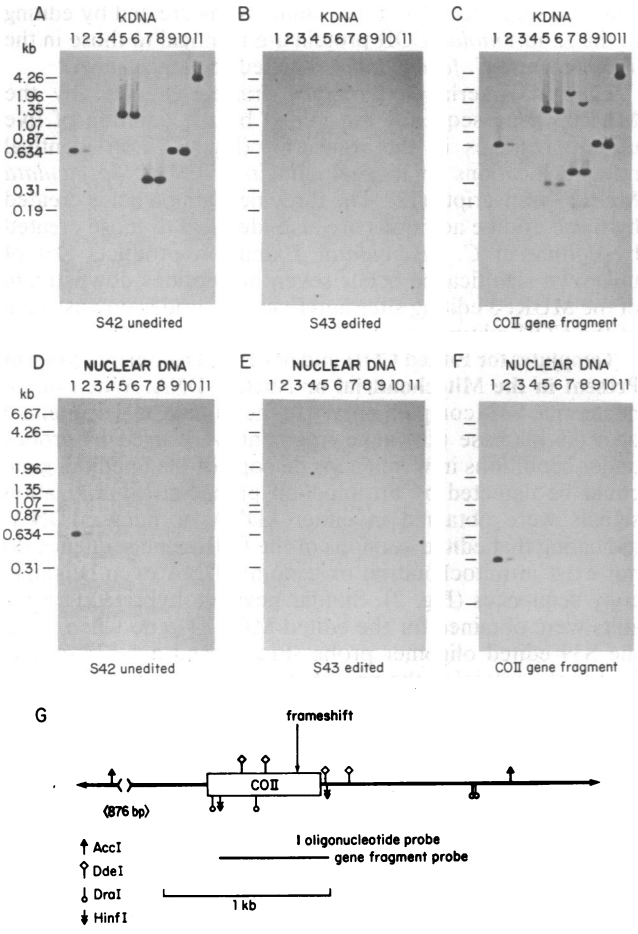


FIG. 2. COII Southern blot analysis of *L. tarentolae* kDNA and nuclear DNA. (*A*, *B*, and *C*) Digested kDNA (10 μg per lane) was electrophoresed on 1.5% agarose gels. Lanes: 5, *Dra* I; 6, *Dra* I and *Acc* I; 7, *Dde* I; 8, *Dde* I and *Acc* I; 9, *Hin*fI; 10, *Hin*fI and *Acc* I; 11, *Acc* I. (*D*, *E*, and *F*) Digested nuclear DNA [1.0 μg (lanes 6, 8, and 10) or 10 μg (lanes 5, 7, and 9)] was electrophoresed on 1% agarose. Lanes: 5 and 6, *Eco*RI; 7 and 8, *Acc* I; 9 and 10, *Dra* I; 11, size marker. The sensitivities of the blots were determined by including the following amounts of a 634-nucleotide *Hin*fI fragment that contains the COII gene: 50 ng (lanes 1), 5.0 ng (100 copies) (lanes 2), 0.5 ng (10 copies) (lanes 3), and 0.05 ng (1 copy) (lanes 4). A signal is visible in lane 4 with longer exposure. Hybridization probes were S42 (*A* and *D*), S43 (*B* and *E*), and COII gene fragment (*C* and *F*).

kRNA or maxicircle DNA as a control and extended in the absence of dGTP through the edited COII region. When kRNA was used as a template, both the predicted 53-nucleotide edited and the 49-nucleotide unedited products were found in a ratio of 8:1, indicating that 90% of COII transcripts exist in the fully edited form. Extension products of intermediate size that could arise from partially edited RNAs were not visible. To determine whether the transcript ratio is similar for a specific uridine addition, the S62 primer was extended in the absence of dATP, dCTP, and dGTP to a position where the 3′-most uridine addition occurs (Fig. 4*B*), with equivalent results.

Primer extension analysis of MURF3 mRNA in the internal edited region gave somewhat different results. A comparison of the ratio of edited to unedited products indicated that only 36% of the MURF3 transcripts were fully edited (Fig. 5*A*). Extension products unique to the RNA template corresponding to partially edited molecules were not detected. Identical results were obtained for the 3′-most uridine addition in MURF3 RNA (Fig. 5*B*).

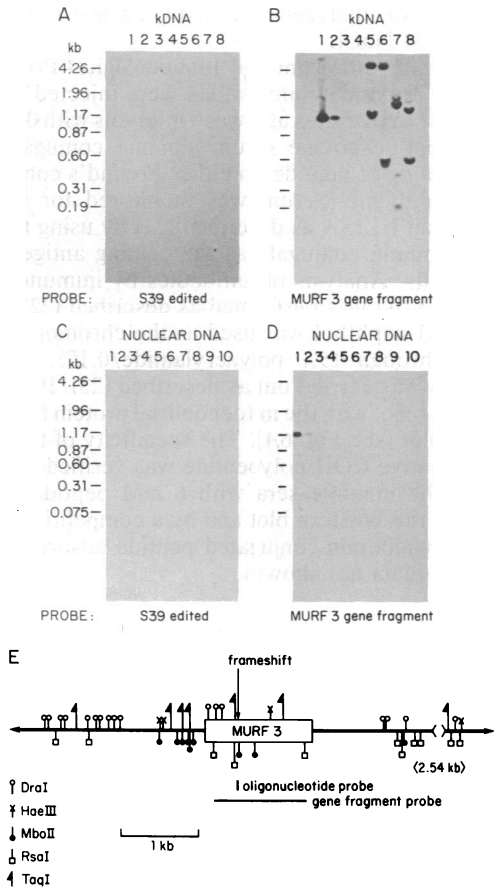


FIG. 3. MURF3 Southern blot analysis of *L. tarentolae* kDNA and nuclear DNA. (*A* and *B*) Digested kDNA (10 μg per lane) was electrophoresed on 1.5% agarose gels. Lanes: 5, *Hae* III; 6, *Taq* I; 7, *Dra* I and *Mbo* II; 8, *Hae* III and *Rsa* I. (*C* and *D*) Digested nuclear DNA [1.0 μg (lanes 6, 8, and 10) or 10 μg (lanes 5, 7, and 9)] was electrophoresed on 1% agarose gels. Lanes: 5 and 6, *Eco*RI; 7 and 8, *Hae* III; 9 and 10, *Rsa* I. The sensitivities of the blots were determined by including the following amounts of a 1.17-kb *Rsa* I fragment that contains the MURF3 gene: 100 ng (lanes 1), 10 ng (100 copies) (lanes 2), 1.0 ng (10 copies) (lanes 3), and 0.1 ng (1 copy) (lanes 4). A signal is visible in lanes 4 with longer exposure. Hybridization probes were S39 (*A* and *C*) and 1.17-kb MURF3 gene fragment (*B* and *D*).

**The Edited COII mRNA Is Translated.** To determine whether the COII RNA is translated, a polyclonal antiserum was generated against a peptide corresponding to the cryptic COOH-terminal sequence of the predicted COII protein. The antiserum reacted with a 21.5-kDa protein (arrow) in Western analysis of a cytochrome-enriched mitochondrial fraction (Fig. 6), which is the predicted molecular weight for the COII polypeptide. The 48-kDa species probably represents a protein aggregate of the COII monomer, since the 21.5-kDa band does not appear in SDS/acrylamide gels (data not shown) but does appear in the more denaturing SDS/urea/acrylamide gels together with a decrease in intensity of the 48-kDa band (Fig. 6). In addition, preincubation of the antiserum with 6 mM COII peptide eliminated staining of both the 48-kDa and the 21.5-kDa species (data not shown).

## DISCUSSION

The internal editing of the *L. tarentolae* COII and MURF3 transcripts involves only uridine additions, and the patterns of additions are strictly conserved between several kinetoplastid species. This differs (*i*) from the complex editing events found within the 5′ ends of mitochondrial transcripts,

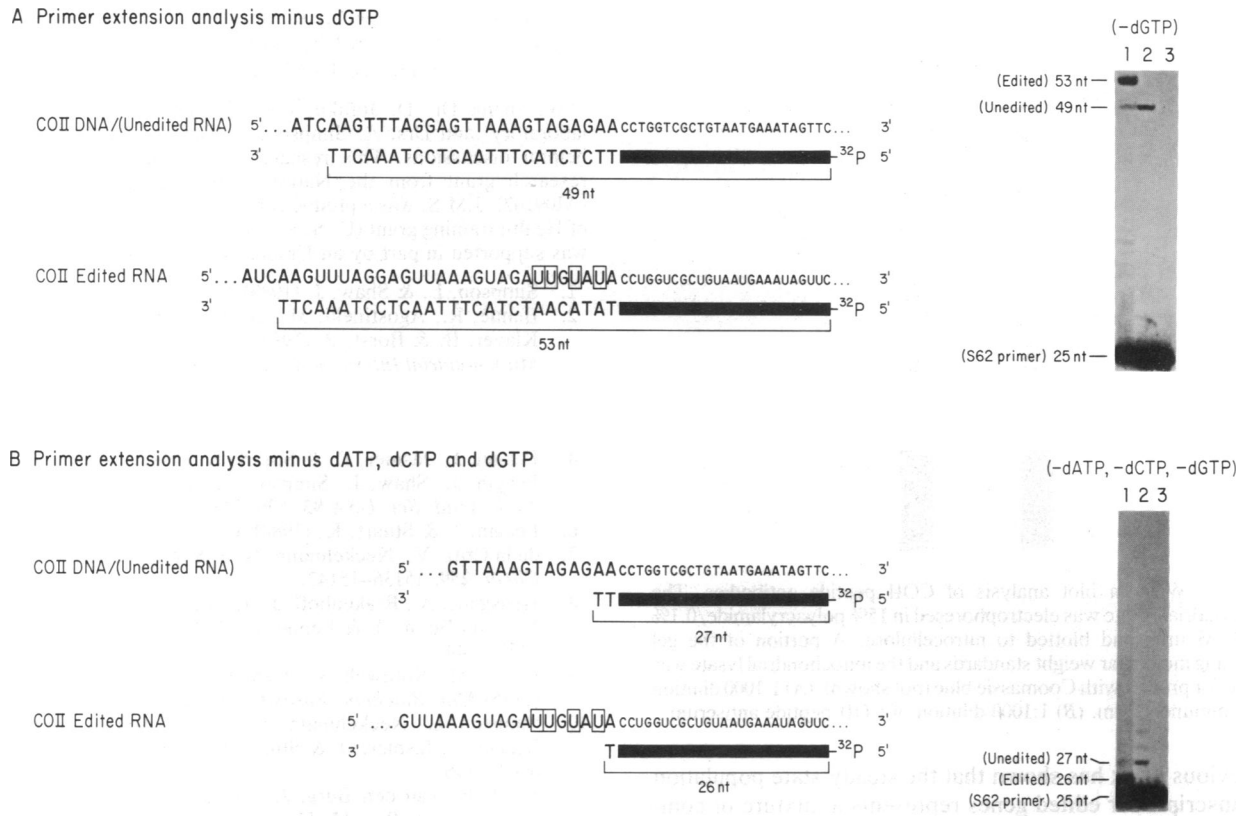


FIG. 4. Primer extension analysis of COII transcripts. The 25-nucleotide (nt) end-labeled S62 primer (black bar) was hybridized to cloned maxicircle DNA or kRNA and extended in the absence of dGTP (*A*) or dATP, dCTP, and dGTP (*B*), and the products were electrophoresed on 15% acrylamide/8 M urea gels. Lanes: 1, kRNA; 2, cloned maxicircle DNA; 3, no template. Sequencing ladders of the COII clone were simultaneously run as size standards (not shown). Added uridine residues are enclosed in boxes.

which involve both uridine additions and, in some cases, deletions and which differ in pattern between species, and (*ii*) from the even more complex panediting (1, 25) events found in the *T. brucei* cytochrome oxidase subunit III mRNA.

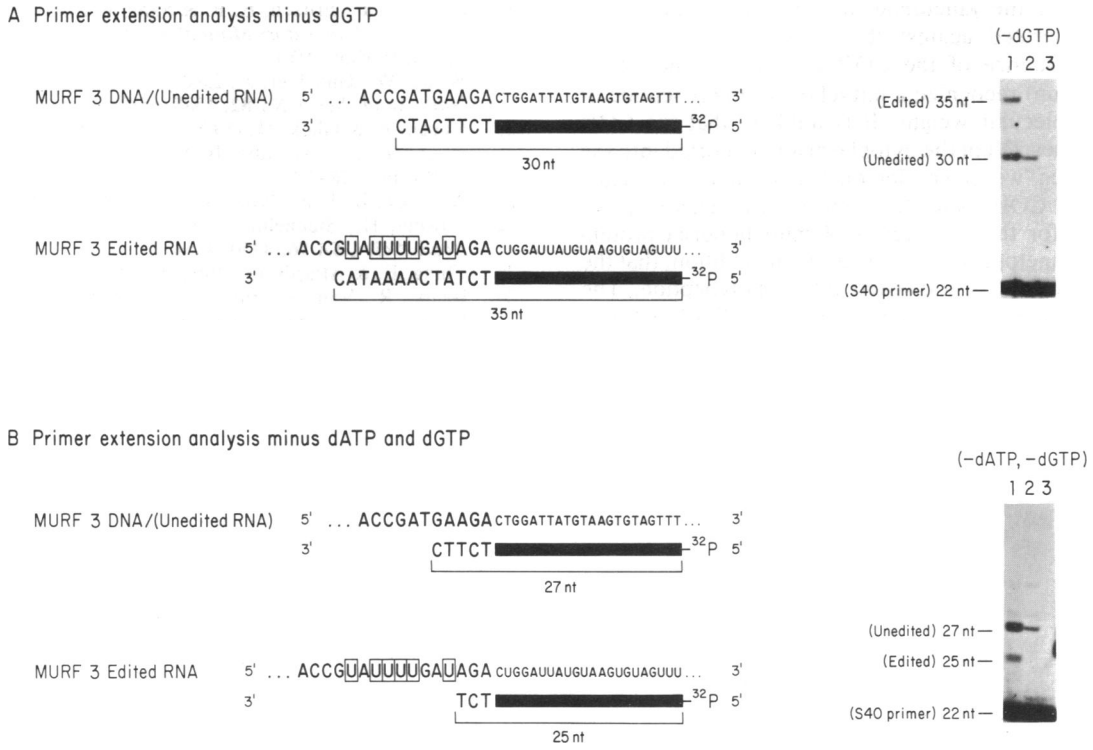


FIG. 5. Primer extension analysis of MURF3 transcripts. The 22-nucleotide (nt) end-labeled S40 primer (black bar) was hybridized to cloned maxicircle DNA or kRNA and extended in the absence of dGTP (*A*) or dATP and dGTP (*B*), and the products were examined by gel electrophoresis. Lanes: 1, kRNA; 2, cloned maxicircle DNA; 3, no template. Added uridine residues are enclosed in boxes.

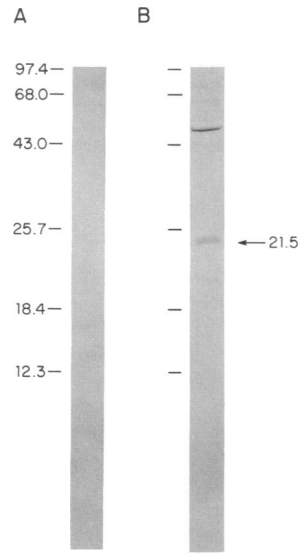


FIG. 6. Western blot analysis of COII peptide antibodies. The mitochondrial lysate was electrophoresed in 15% polyacrylamide/0.1% SDS/8 M urea and blotted to nitrocellulose. A portion of the gel containing molecular weight standards and the mitochondrial lysate was stained for protein with Coomassie blue (not shown). (*A*) 1:1000 dilution of preimmune serum. (*B*) 1:1000 dilution of COII peptide antiserum.

Previous work has shown that the steady-state population of transcripts for edited genes represents a mixture of completely edited, unedited, and partially edited molecules (3–6, 12, 24, 25). Our results indicate that the ratio of edited to unedited RNA varies from gene to gene in the steady-state RNA population. In addition, partially edited transcripts of either gene are undetectable by this method and are therefore either absent or present in low abundance.

Mitochondrial protein synthesis has never been rigorously demonstrated in the kinetoplastids. We have shown that antibodies generated against the cryptic COOH-terminal amino acid sequence of the COII gene (3′ of the edited frameshift region) recognize a mitochondrial polypeptide of the correct molecular weight. It is unlikely that the COII protein is synthesized in the cytoplasm and imported into the organelle, since we have demonstrated that a nuclear-encoded edited COII template is not present. This is therefore evidence for the occurrence of mitochondrial protein synthesis in a kinetoplastid and suggests, in addition, that the edited COII mRNA is translated into a polypeptide. The possibility that unedited mitochondrial COII RNA is translated via a mechanism such as ribosomal frameshifting cannot be ruled out in the absence of amino acid sequence data in the region of the frameshift but appears unlikely given the low abundance of unedited COII transcripts.

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