The term RNA editing encompasses two types of specific alterations in the coding potential of RNA molecules: base substitution and the insertion (or deletion) of nucleotides. Such changes in RNA sequence can have profound effects on gene expression, and, indeed, most genes in the mitochondria of plants, trypanosomatids, and *Physarum polycephalum*. Editing of this mRNA includes the insertion of cytidine, guanosine, and uridine residues, as well as the apparent conversion of cytidines to uridines. No edited version of this gene was detected in *Physarum* DNA, and amino acid alignments suggest that both types of RNA editing are required to produce a functional protein.

RNA editing via the insertion of nucleotides has been observed in the mitochondria of trypanosomatids (1-4) and *Physarum polycephalum* (5, 6). In *Physarum*, the mRNA encoding the α subunit of the ATP synthetase (α ATPase) is altered via the specific addition of 54 single cytidine (C) residues (6). The source of the added information is unknown. Although it has been proposed that the multiple uridine (U) insertions that occur in trypanosomatid mitochondria are mediated by guide RNAs (7), there is currently no evidence that guide RNAs exist in *Physarum* mitochondria. The identity and spacing of added nucleotides and the lack of nucleotide deletions also argue that a unique editing mechanism may be utilized by *P. polycephalum*. We demonstrate here that this system is quite complex, as multiple types of RNA editing events are taking place in *Physarum* mitochondria.

**EXPERIMENTAL PROCEDURES**

**DNAs and RNA**—A cloned 5.5-kb *XbaI* fragment of *Physarum* mitochondrial DNA (pPH1202, 8) was generously provided by Dennis Miller. A subcloned 2.1-kb *HindIII* fragment derived from this plasmid hybridized to mitochondrial RNA on Northern blots and was sequenced in its entirety. Mitochondrial DNA and RNA were isolated from *Physarum* strain M.C. using the procedure of Jones et al. (8). cDNAs were made using col-specific primers and avian myeloblastosis virus reverse transcriptase (Life Sciences) in a buffer containing 29 mM Tris-HCl (pH 8.3), 125 mM KCl, 16 mM MgCl₂, 5 mM dithiothreitol, and 400 μM each dNTP at 42 °C. Sequencing—Multiple, independent PCR clones derived from mtDNA or cDNAs were sequenced using the Sequenase kit (U. S. Biochemical Corp.). Bulk *Physarum* mtRNA was sequenced with reverse transcriptase under conditions recommended by the manufacturer using 200 μM dideoxynucleotides for chain termination. PCR—30 cycles of PCR (1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C) were carried out in 10 mM Tris-HCl (pH 8.5 at 25 °C), 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin, 50 μM dNTPs, 1 μM of each primer, and 2.5 units of Taq polymerase to generate col fragments for cloning or restriction analysis.

**Oligonucleotides**—For restriction digests of end-labeled PCR products, the upstream primer (5'-labeled 5'-CTGGAACTGTTGG-GACTG-3') was 5'-end-labeled using [γ³²P]ATP and polynucleotide kinase (Boehringer Mannheim), and the downstream primer (5'- TAAAGCCACCTAATGTTAAAC-3') was unlabeled. Oligonucleotides N (5'-ACATTCTGGATGGCCTTACCTC-3'), U (5'-AAATAAGCGCGATGATCCA-3'), and E (5'-AATAAGCGCGATGATCCA-3') were used as Southern hybridization probes.

**Southern Hybridization Analysis**—DNA fragments were separated via gel electrophoresis (1% agarose, 40 mM Tris-acetate (pH 8), 1 mM EDTA) and transferred to GeneScreen (DuPont NEN). Oligodeoxynucleotides N, E, and U were 5'-end-labeled and hybridized to filters overnight at 37 °C in 0.5 M sodium phosphate (pH 7.2), 2 mM EDTA, 7% SDS, and 1% BSA. Filters were washed at 45 °C in 5 x SSC and 0.1% SDS, then in 1 x SSC and 0.1% SDS (1 x SSC = 0.15 M NaCl, 0.01 M sodium citrate, pH 7). All enzymes were used under conditions recommended by the manufacturer.

**RESULTS AND DISCUSSION**

The mitochondrial genome of *Physarum* is approximately 60 kb in length (8). We have focused our studies on a previously uncharacterized 2.1-kb region of the genome that is known to be actively transcribed (Ref. 8; data not shown). Sequencing of mitochondrial DNA (mtDNA) revealed that this region is highly homologous to genes for subunit I of cytochrome oxidase (col) from a variety of mitochondria. However, protein alignments indicated that numerous frameshifts would be required to produce a functional Col protein, suggesting that the *Physarum* Col mRNA is edited. Using oligonucleotide primers that hybridize to regions that are not predicted to be edited (nonedited), we generated cDNAs by reverse transcription of mitochondrial RNA (mtRNA), then amplified these cDNAs, as well as fragments of mtDNA, and total *Physarum* DNA using PCR.

As anticipated from our preliminary sequence data, mtDNA and mtRNA (cDNA) col sequences differ by the addition of nucleotides at predicted frameshift sites (Fig. 1). Most, but not all, are single C insertions (59/64 editing sites; Fig. 2). The distribution and context of C insertions are similar to those of the α ATPase mRNA (6), with irregular spacing (13-125 nucleotides) between added C nucleotides and a strong bias for insertion after a purine-pyrimidine dinucleotide. Remarkably, there are also insertions of a single U residue and 2 dinucleotides, C

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1 The abbreviations used are: kb, kilobase pair(s); PCR, polymerase chain reaction; mtDNA, mitochondrial DNA; mRNA, mitochondrial RNA.
Physarum and colleagues viral sequencing of total mtRNA using reverse transcriptase and an end-Product derived from mtDNA and cDNAs made by reverse transcription of total mtRNA was confirmed via primer extension sequencing of total mtRNA. A region that includes four C insertions was present in the bulk mtDNA and cDNAs. This was unexpected, as there are no substitutions and three insertions of nucleotides other than G, and U changes are clearly right, primer extension (*), insertion (**). Brackets indicate equivalent regions of each panel.

Both the base substitutions and the insertion of C, G, and U residues into the Physarum col mRNA are likely to be functional, as the deduced protein sequence is highly homologous to col proteins from a variety of species (Fig. 3). Interestingly, one of the codons created by a C → U substitution has also been suggested to be a site of change in the col mRNA from primrose mitochondria (14).

Fig. 2. Coding sequence of the edited col mRNA. Sequence is a composite from overlapping cDNA clones derived from Physarum mtRNA. Residues that are the result of RNA editing are underlined and in boldface type. Uppercase letters represent nucleotides that are encoded in the DNA, with the C → U changes represented by capital, bold, underlined “U.” Lowercase letters are inserted nucleotides. Where the sites of insertion are ambiguous, the added nucleotides are arbitrarily placed at the 3' side of the site.

Fig. 3. Alignment of the deduced amino acid sequence of the Physarum col protein in the region spanning the editing sites shown in Figure 1 with the equivalent region of col from the mitochondria of wheat, evening primrose, sea urchin, rat, cow, Drosophila, and Xenopus and the large subunit of the bacterial cytochrome c oxidase (cytochrome aa3) from Paracoccus denitrificans. WHT, wheat (18); OBS, evening primrose (19); PAL, sea urchin (20); RAT, rat (21); Bov, cow (22); DRO, Drosophila (23); XEL, Xenopus (24); PAR, large subunit of bacterial cytochrome c oxidase (cytochrome aa3) from P. denitrificans (25). +, codon created by U insertion; *, codons formed by C insertion; †, codons present after C → U substitutions. Lowercase letters in the evening primrose sequence indicate encoded residues predicted to be altered by RNA editing (14).
Assays for the presence of an edited col gene. A, Southern hybridization analysis. Restriction sites for HindIII (H), DraI (D), PvuII (P), and MspI (M) and the sizes of DNA fragments expected to hybridize to oligonucleotide probes N (nonedited), U (unedited), and E (edited) are shown schematically at top. Probes U and E overlap the site of the CU dinucleotide insertion. DNA was digested with the following: a, HindIII; b, HindIII + PvuII; c, DraI +PvuII ± HindIII; d, DraI + PvuII + MspI ± HindIII. B, restriction digestions of end-labeled PCR products. Cloned mtDNA (unedited), cloned cDNA (edited), bulk cDNA synthesized from mtRNA, mtDNA, and total Physarum DNA were used as templates for PCR using as primer oligodeoxynucleotides that should anneal to both edited and unedited sequences. End-labeled PCR products were digested with either DraI or MspI and separated via electrophoresis through a 5% acrylamide, 7 M urea gel.
assay. Based on the sequence of the mtDNA and cDNA clones, an edited version of the gene would have a restriction map that differs significantly from the unedited copy. Two of these differences, sites for DraI and MspI, were exploited in experiments designed to detect a second col gene. To further increase sensitivity, we utilized three different oligonucleotides as hybridization probes (indicated schematically at the top of Fig. 4A). The first probe hybridizes to a region of the col gene that is not edited (nonedited, N), the second oligonucleotide is complementary to the unedited gene (U), and the third probe is specific for edited sequences (E).

As shown in the Southern blots presented in Fig. 4A, no edited version of the col gene was detected. An edited gene should give a pattern identical to the cDNA (edited) clone (lanes 1 and 2), while the unedited version of the gene should give a pattern identical to the cloned mtDNA (lanes 3 and 4). Only the unedited pattern was observed with either mtDNA (lanes 5–8) or total Physarum DNA (lanes 9–12) with probes hybridizing to nonedited or unedited regions. In addition, no hybridization of the probe specific for edited sequence was observed to either mtDNA or total DNA under conditions that give a strong signal with the cDNA clone. Similar results have been obtained with other restriction enzymes and other oligonucleotide probes that are specific for edited or unedited sequences (data not shown).

A related strategy was employed for a PCR assay in which one of the primers was end-labeled and the radiolabeled PCR fragments were digested with either DraI or MspI (Fig. 4B). Digests of PCR products derived from mtDNA and total DNA resembled that from an unedited mtDNA clone, yielding evidence that no edited version of the unambiguous sequence, indicating that the bulk of the RNA is homogeneous and edited. 2) Restriction digests of PCR products derived from bulk or cloned cDNAs yielded a 303-base pair DraI fragment (lanes 4, 7, and 8) and lacking restriction sites for MspI (lanes 9, 12, and 13). Conversely, PCR products derived from bulk or cloned cDNAs yielded a 303-base pair MspI fragment (lanes 10 and 11) and were undigested by DraI (lanes 5 and 6). These experiments provide strong evidence that no edited version of the col gene is present in P. polycephalum.

Three lines of evidence support the idea that editing of the Physarum col mRNA is a highly efficient and accurate process. 1) Primer extension sequencing of total mRNA (Fig. 1) gives an unambiguous sequence, indicating that the bulk of the RNA is homogeneous and edited. 2) Restriction digests of PCR products derived from bulk col cDNA (Fig. 4B, lanes 6 and 11) suggest that virtually all of the mRNA is edited. 3) The oligonucleotide probes used in Fig. 4A have also been used to probe Northern blots of total mitochondrial RNA. Probes complementary to nonedited and edited RNAs each hybridized to an RNA of approximately 1900 nucleotides, while the oligonucleotide specific for unedited sequences did not hybridize to RNA (data not shown). Taken together, these data suggest that Physarum editing is either a co-transcriptional process or that transcription and editing are tightly coupled in Physarum mitochondria. Further work will be needed to distinguish between these mechanistic alternatives. Nevertheless, the existence of both substitutional and insertional RNA editing in a system that is amenable to biochemical, developmental, and genetic analysis (15–17) should facilitate the study of multiple editing mechanisms.

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