The reading frame in the mRNA for the cytochrome b apoprotein in mitochondria of *Physarum polycephalum* is created by the insertion of 43 nucleotides in the mRNA relative to the mtDNA sequence encoding it (RNA editing). Most of these insertions (31) are single cytidines; however, single uridines are inserted at six sites, and the dinucleotides, CU and GC, are inserted at two sites and one site, respectively. These insertions create a 392-codon reading frame in the mature mRNA. The amino acid sequence inferred from this reading frame has similarity to cytochrome b apoproteins encoded by other mtDNAs. The insertions are quite evenly distributed throughout the length of the reading frame with an average spacing of 27 nucleotides. This mRNA has the highest percentage (23%) of noncytidine insertions of average spacing of about 25 nucleotides in mRNA and 45 nucleotides in rRNA. In this organism about 25% of the nucleotides in rRNA. In *Physarum* mitochondria, RNA editing has been observed in all of the three RNA types: mRNA (2, 5, 6), tRNA (9), and rRNA (7). RNA editing is required for gene expression because it produces a continuous open reading frame in mRNAs and produces conserved primary sequences and secondary structures in tRNAs and rRNAs.

In contrast to trypanosome editing in which only uridines are inserted (10), in *Physarum* mitochondria any of the four nucleotides can be inserted but only in certain combinations (2, 5–9). Cytidines and uridines can be inserted as single nucleotides or members of certain dinucleotides (for example, AU, GU, and CU). Adenosine and guanosine have only been observed to be inserted as members of a dinucleotide insert (for example, AA and AU for adenosine and GU for guanosine).

The fact that dinucleotide insertions are not just adjacent single nucleotide insertions is indicated by both editing site distribution and by the composition of the dinucleotide inserts. If dinucleotide insertions were produced by the occasional placement of two single insertion sites at adjacent positions, then the composition of the dinucleotide sites would reflect the composition of the single nucleotide insertion sites. This is clearly not the case because adenosine and guanosine nucleotides have only been observed to be inserted at dinucleotide insertion sites and because CC insertions, the type of dinucleotide insertion predicted to be most common based on single insertion nucleotide frequency, are not normally observed in *Physarum* RNA. In addition, analysis of editing site distribution reveals an apparent constraint on how close together single nucleotide editing sites can be. No two sites have been observed to be separated by fewer than nine nucleotides (3). In terms of their relationship to single nucleotide insertions, dinucleotides could be inserted in several ways. For example: 1) All nucleotides could be inserted by the same process, independent of their identity or whether they are inserted as mononucleotides. 2) Nucleotides could be inserted by type at specific sites, such that one type of nucleotide is first inserted at a heterodinucleotide insertion site and then a second nucleotide is inserted to complete the site. This would also predict that single cytidines would be inserted separately from single uridines. The order in which nucleotide types are inserted could be sequential or random. 3) Dinucleotides could be inserted in a separate process from the insertion of mononucleotides. This would predict that cytidines and uridines in heterodinucleotides would be inserted separately from the insertion of cytidines or uridines as single nucleotides.

RNA editing in *Physarum* is very efficient as judged by the frequency at which insertions are present at potential editing sites in steady state RNA (3–6, 8). In the mRNA of ATP synthase, the frequency of editing sites without a nucleotide insertion is lower than 5% (3–5). One possible explanation for this efficiency is that editing in *Physarum* occurs at or very near the mitochondrial transcription complex so that nascent transcripts are edited as an early step in mitochondrial gene expression. Consistent with this idea Visomirski-Robic and
Gott (11, 12) have shown that in isolated mitochondria in which one of the ribonucleotide triphosphates is limiting, RNA editing has occurred very close to the stalled mitochondrial RNA polymerase. However, cDNAs corresponding to RNAs that are partially edited have been detected (9, 12). Whether these RNAs are RNA editing intermediates or aberrant, incompletely edited by-products of the editing process, their nucleotide insertion pattern provides information about the way in which nucleotides are inserted in Physarum mitochondrial RNAs. Because these RNAs are rare, cDNAs corresponding to them have been difficult to isolate.

We report here that the Physarum mitochondrial apocytochrome b mRNA is extensively edited. This editing includes not only single cytidine insertions but also a significant number of single uridine insertions as well as three dinucleotide insertions, one of which (GC) has not been previously reported. We also describe a method to isolate and examine PCR-derived cDNAs of very rare, partially edited mRNAs that provide information about the relationship of dinucleotide and mononucleotide insertions.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—** *P. polycephalum* strain M3 (originally characterized at McArdle Laboratory, Madison, WI) was generously provided by R. Marsh (University of Texas at Dallas, Richardson, TX) from the Guttes collection. Microplasmodia were grown at 27°C in the dark, with continuous agitation, in semi-defined *Physarum* medium (13). Microplasmodia were generally harvested 96 h after inoculation.

**Isolation of Mitochondria—** mtDNA was isolated from microplasmodia as described by Jones et al. (14). After microplasmodia were harvested by centrifugation and washed with water three times, they were homogenized in a Waring blender for 15 s. The homogenate was centrifuged at 650 × g for 5 min to pellet nuclei and cell debris. Mitochondria were pelleted from the supernatant fluid by centrifugation at 8000 × g. The mitochondria were then purified by several rounds of differential centrifugation. Purified mitochondria were used for RNA and DNA isolation.

**RNA Isolation—** All solutions for the isolation of RNA were prepared in diethylpyrocarbonate-treated water. RNA was isolated from mitochondria (15) by lysis in an equal volume of lysis buffer (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.2% SDS) followed immediately by three rounds of phenol extraction and one chloroform extraction. The aqueous layer was separated from the organic phase by centrifugation at 12,000 × g for 10 min. RNA was then precipitated with 0.3 volumes of 2 M potassium acetate, pH 4.55, and 3 volumes of ethanol and stored at −20°C.

**Northern Hybridization Analysis—** mtRNAs were separated by electrophoresis on a 1.5% agarose, 7 M urea gels. The gel was electroblotted in 20 mM HCl, pH 8.0, 2 mM EDTA, 0.2% SDS) followed immediately by three rounds of phenol extraction and one chloroform extraction. The aqueous fraction about the relationship of dinucleotide and mononucleotide insertions. Insertion pattern provides information about the way in which edited by-products of the editing process, their nucleotide insertions have been difficult to isolate.

**RESULTS AND DISCUSSION**

We have studied a previously uncharacterized region of the *Physarum* mitochondrial genome that is known to be actively transcribed (14). Radiolabeled probes from this region detect a distinct 1.7-kb RNA band (Fig. 1). The mtDNA sequence of the...
region coding for this RNA does not contain a significant open reading frame. However, small stretches of open reading frame encode amino acid sequences that are similar or identical to the sequence of cytochrome \( b \) (\( cyt \ b \)) from a variety of mitochondria. Amino acid sequence alignments indicated that numerous frameshifts were required to produce one continuous open reading frame with homology to \( cyt \ b \), suggesting that the \( Physarum \) mitochondrial \( cyt \ b \) mRNA is edited. To test whether this was the case, cDNAs were generated from mtRNA using reverse transcriptase and then amplified using PCR. Comparison of cDNA and mtDNA sequences reveals that the mtRNA sequences differ from their mtDNA template by the addition of nucleotides that compensate for the frameshifts (Fig. 2). Totally, there are 40 insertion sites. Most (31 of 40) are single C insertions. However, 6 single U insertions as well as two types of dinucleotide insertion (CU and GC) are also observed. Although AU, CU, and GU insertions have been previously observed in the mRNA for subunit 1 of cytochrome oxidase (6, 8), and CU and AA dinucleotide insertions have been previously reported in the small subunit rRNA of \( Physarum \) mitochondria (7), this is the first observation of a GC dinucleotide insertion. This GC dinucleotide insertion provides a second way in which guanosines can be inserted in \( Physarum \) RNA and, taken together with the previous observation of AA and AU dinucleotide insertions (6, 8) and C and U insertions (2, 5–7, 9), indicates that any of the four nucleotides can be in-

FIG. 2. Alignment of the mtDNA and cDNA sequences of \( cyt \ b \). The top line is the mtDNA sequence, and the center line is the cDNA sequence. Gaps are inserted in the mtDNA sequence to maximize sequence alignment. The numbers indicate the location of nucleotide insertions in the cDNA sequence relative to the mtDNA sequence. The amino acid sequence deduced from the open reading frame in the cDNA sequence is shown below the cDNA sequence. The locations of the oligonucleotide primers used in the synthesis of cDNAs are indicated by the underlined sequences. Primer designations are indicated in bold type above the underlined sequence. Restriction enzyme sites in parentheses are absent in the mtDNA but present in the cDNA due to their creation by nucleotide insertion.
that editing sites are confined to functional sequences in insertion sites in the small subunit rRNA (SSU, Ref. 7), the cox1 mRNA (Col, Ref. 6), and tRNA^K (tRNA-K and tRNA-E, Ref. 9). Sequences are aligned to emphasize the potential of uridine insertion downstream of purine-pyrimidine dinucleotides. R, Y, and + indicate the purine, the pyrimidine, and the potential uridine insertion site used for the alignment. The inserted uridine and uridines flanking the insertion site are underlined.

All three dinucleotide insertions are at sites flanked by one or more nucleotides that are the same as the inserted nucleotides. This results in a cDNA sequence in which it is ambiguous which of several nucleotides corresponds to the inserted nucleotide. This also makes it ambiguous whether the dinucleotide insertion is GC or CG, CU or UC. This ambiguity has been observed for other dinucleotide insertions as well (6–8). Insertion is GC or CG, CU or UC. This ambiguity has been observed for other dinucleotide insertions as well (6–8).

Editing Site Distribution—The editing sites are relatively evenly distributed throughout the entire coding sequence with an average separation of 27 nucleotides and a standard deviation of 15 nucleotides (Fig. 2). As observed for other edited RNAs in Physarum (2, 6, 7, 9), there is a bias for cytidine insertions to be downstream of purine-pyrimidine dinucleotides. The inserted uridine and uridines flanking the insertion site are underlined.

Analysis of the cyt b mRNA indicates that editing sites are confined to the open reading frame. Although the average spacing between editing sites is 27 nucleotides within the reading frame, no editing sites were detected within 85 bases of the termination codon or within 65 additional bases downstream of the termination codon. Nor were any editing sites detected for greater than 100 nucleotides upstream of the initiation codon. However, a reading frame is not required for editing because RNAs that lack significant open reading frames are also edited in Physarum (7, 9). This may instead indicate that editing sites are confined to functional sequences in Physarum mtRNAs.

Uridine Insertion Sites—At six sites (sites 10, 18, 22, 24, 26, and 40) single uridines are inserted. Sequence analysis of numerous independent cDNAs consistently showed uridine and not cytidine insertions at these sites. Although a single uridine insertion has been reported in the mRNA for subunit 1 of cyt b Reading Frame—Insertion of these 43 nucleotides creates an open reading frame of 392 codons. Sixteen different codon types are created by C insertions, and four codon types are created by U insertions. Of the nine C insertion codon types in which the location of the insertion can be unambiguously assigned, four (CUU, CUA, CAA, and CGU) have the C insertion at the third position. The four codon types in which cytidine is inserted at the third position are used eight times and make up 62% of the 13 cytidine insertions in which the location of the insertion can be unambiguously assigned. The relative position of the U insertions within the codon cannot be determined because, with one exception, the insertions are next to a pre-existing U.

The amino acid sequence deduced from the open reading frame in the cDNA sequence shows extensive homology to the cyt b apoprotein from mitochondria of yeast (19), man (20), and maize (21) (Fig. 4). All of the major amino acid motifs conserved in plants, animals, and fungi are also present in the sequence inferred from the edited RNA from Physarum. These data indicate that the 1.7-kb RNA is the apocytochrome b mRNA and that it is extensively edited to generate a functional mRNA. Consistent with the ancient divergence of Physarum from the eukaryotic line, the Physarum sequence is as divergent from the cyt b sequences of plant, animals, and fungi as these sequences are from one another.

The GC Dinucleotide Insertion Is a Separate Event from Single Cytidine Insertions—To determine the relationship of the GC dinucleotide insertion to the more prevalent single cytidine insertions, a protocol has been developed to isolate and examine cDNAs corresponding to partially edited transcripts. The insertion of the GC dinucleotide in mRNA creates an AluNI site in its cDNA. If the GC dinucleotide is not inserted

![Fig. 3. Alignment of uridine insertion sites. The six sites of single uridine insertion in cyt b mRNA are aligned along with the uridine insertion sites in the small subunit rRNA (SSU, Ref. 7), the cox1 mRNA (Col, Ref. 6), and tRNA^K (tRNA-K and tRNA-E, Ref. 9). Sequences are aligned to emphasize the potential of uridine insertion downstream of purine-pyrimidine dinucleotides. R, Y, and + indicate the purine, the pyrimidine, and the potential uridine insertion site used for the alignment. The inserted uridine and uridines flanking the insertion site are underlined.](image)
or if only one of the two nucleotides is inserted, this site will be absent in cDNAs derived from this RNA (Fig. 2). Because the AlwNI recognition sequence (5'-CAGNNNCTG-3') includes six specific nucleotides and spans nine nucleotides, any of a number of substitutions, deletions, or insertions would result in the absence of the AlwNI site in the cDNA. To enrich for rare cyt b cDNAs lacking the AlwNI site, cDNAs produced by RT-PCR from cyt b mRNA that lacked the AlwNI site were selectively amplified. A mtRNA preparation was first digested with DNase I to remove contaminating mtDNA and then used as a template for reverse transcriptase to synthesize cDNAs. PCR primers flanking several editing sites including the GC insertion site were used to selectively amplify cDNAs derived from the mtRNA. These amplification products were digested with AlwNI, and the two digestion products were separated from the uncut amplification product by electrophoresis on a 1% agarose gel. Almost all of the amplification product was digested by AlwNI. This result indicates that a high percentage of mtRNAs in the steady state population is edited at this site, consistent with previous results from Gott et al. (6, 8) and Miller et al. (3–5) indicating that the efficiency of nucleotide insertion at a given editing site is greater than 95%. The undigested amplification product was excised from the agarose gel, and the residual DNA was isolated. The isolated DNA was reamplified using the same primers. The resulting PCR products were digested with AlwNI a second time to eliminate amplification products derived from incomplete digestion in the first round. The undigested DNAs were cloned and sequenced.

In each trial some amplification products were cloned which lacked insertions at any position. Whether these clones derive from unedited RNAs or from residual mtDNA which escaped DNase I digestion is not known. However, three independent amplification products which lacked the AlwNI site did have insertions at editing sites. With the initial primer set whose amplification products spanned 10 editing sites, we obtained two independent cDNA clones which were fully edited at each of the single cytidine insertion sites but lacked the GC dinucleotide insertion (cDNAs 1 and 2, Fig. 5).

An additional cDNA clone (cDNA 3, Fig. 5) was obtained using a different RNA preparation and a different set of primers that spanned 22 insertion sites including 16 single C insertions, two single U insertions, and two CU dinucleotide insertions. This cDNA also lacked the GC insertion but was otherwise fully edited. These results suggest that the cytide
of the GC dinucleotide insertion is not added by the same process in which single cytidines are inserted and that the GC dinucleotide insertion occurs separately from mononucleotide insertions.

The CU Dinucleotide Insertion Is a Separate Event from Single Nucleotide Insertions—Insertion of the CU dinucleotide was examined using a similar method. Insertion of the CU dinucleotide at site 11 in cyt b mRNA creates a \( MnlI \) site in its cDNA (Fig. 5). This \( MnlI \) site is absent in cDNAs that lack one or both of the inserted nucleotides. Primers were used that produced an amplification product spanning the first 14 editing sites. An amplification product (cDNA 4) had insertions at editing sites immediately adjacent to the CT dinucleotide insertion site but lacked the CT dinucleotide insertion. This cDNA also lacked a C insertion at site 6.

A second round of selection using a different RNA preparation produced four unique cDNAs that lacked the \( MnlI \) site (cDNAs 5–8). Similar to cDNA 4, cDNA 5 lacked the CT insertion but was fully edited at sites adjacent to the CT insertion site. In addition seven clones had the editing pattern shown for cDNA 6, i.e. the CT of site 11 was missing as well as the C at the adjacent editing site (site 12) located 19 nucleotides downstream of the CU editing site. Two amplification products had dinucleotide insertions that did not create the \( MnlI \) site. In cDNA 7 the dinucleotide insertion was CC, and in cDNA 8 the dinucleotide insertion was TT.

Results suggest that the cytidine and uridine of the CU dinucleotide insertion are not added by the same processes in which single cytidines or single uridines are inserted and that the CU dinucleotide insertion occurs separately from mononucleotide insertions.

Selection for cDNAs That Lack the Styl Site—A Styl site is created in cDNAs by a cytidine insertion at site 14. Selection for cDNAs that lack this site using the protocol described above produced amplification products that lacked the Styl site. These cloned amplification products were screened for the presence of the \( AlwNI \) or \( MnlI \) site, but none of the cloned amplification products that lacked the Styl site had either the \( AlwNI \) or \( MnlI \) site, indicating that they lacked the GC and CU dinucleotide insertions. Sequence analysis of 21 of these cloned amplification products revealed sequences that corresponded to fully unedited RNAs. Two types of cDNA corresponding to partially edited RNAs that might be expected based on the dinucleotide experiments were not detected, i.e. cDNAs in which only one or both of the dinucleotide insertions is present in otherwise unedited context (\( Sty^+ \), \( MnlI^+ \), and/or \( AlwNI^+ \)) and cDNAs in which a cytidine is not inserted at site 14 in otherwise fully edited context (\( Sty^+ \), \( MnlI^+ \), and \( AlwNI^+ \)). Failure to detect the latter type, in contrast to the ease of isolating the analogous cDNA for a dinucleotide site (18 partially edited sequences of 24 cDNAs selected for \( MnlI^+ \) using the same RNA preparation and primer set for RT-PCR), provides additional evidence that mononucleotide and dinucleotide insertions are produced by a separate process. Furthermore, these results indicate that the cDNAs reflect the RNA population and are not an artifact of the selection process. That is, cDNAs corresponding to RNAs with certain editing patterns cannot be selected presumably because they are not represented in the RNA population.

Dinucleotide Insertions—Analysis of cDNAs corresponding to partially edited RNAs indicates that dinucleotide insertions are independent from cytidine insertions in that dinucleotide insertions can be absent in sequences fully edited at single cytidine and uridine sites. This observation is consistent with the unique base composition of the dinucleotide insertions and implies at least some degree of autonomy from the process of single cytidine insertion.

Visomirski-Robic and Gott (11, 12) have shown that in isolated mitochondria provided with limiting concentrations of one exogenous ribonucleotide triphosphate, nucleotide insertions can be detected in nascent RNAs at both single nucleotide and dinucleotide editing sites located very close to the mitochondrial transcription complex. Although it is clear that under some conditions editing can be closely associated with the transcription complex, it is not clear whether editing is cotranscriptional with nontemplated nucleotides added to the 3’ end of the nascent RNA or post-transcriptional with nontemplated nucleotides inserted between two templated nucleotides in the RNA.

If editing is post-transcriptional in this sense, then it is likely that the partially edited RNAs are intermediates that have not yet been completely edited. The fact that sequences were identified that have cytidine insertions but lack dinucleotide insertions, but not the converse, implies that GC insertions may occur after global cytidine insertion. If editing is cotranscriptional, then partially edited cDNAs must derive from RNAs in which editing failed to insert a nucleotide at a potential editing site. These RNAs would not be intermediates but would indicate that dinucleotide insertions can be skipped without affecting subsequent cytidine insertion. A third possibility is that cytidine insertions are cotranscriptional, whereas dinucleotide insertions are post-transcriptional, which would again indicate that dinucleotides are inserted by a separate mechanism from cytidine insertions.

We did not detect a single nucleotide inserted at dinucleotide insertion sites, indicating that the two nucleotides of a dinucleotide insertion are inserted at about the same time under normal growth conditions. Visomirski-Robic and Gott (12) have reported that RNA synthesized in isolated mitochondria that were incubated under conditions of low ribonucleotide triphosphate concentrations can produce partially edited RNAs similar to the type selected by our technique. They have reported that at low concentrations of UTP, RNAs are produced that can lack the U at a CU insertion site but have the C inserted at this site. This implies that UTP is probably the source of the uridine insertions in heterodinucleotides as CTP is for single cytidine insertion sites. Significant amounts of RNAs with a single
nucleotide insertion at potential dinucleotide editing sites may occur only under conditions in which a source ribonucleotide triphosphate is limiting. In contrast, under conditions of limiting CTP, which result in partially edited RNAs with C insertions missing at potential single cytidine editing sites, the CU dinucleotide editing site has both nucleotides inserted (12). The fact that CU dinucleotide and single C nucleotide sites are differentially affected by low CTP concentrations is consistent with the editing of these two types of site being mechanistically separate.

Two cloned cDNAs that lacked the MnlI site had nucleotide insertions but of the wrong type to create the restriction site. Instead of the CT insertion found in most cDNAs at this site, these two cDNAs had a CC or a TT insertion at the site. It is not known whether these cloned cDNAs represent aberrant RNAs in which the wrong nucleotide was inserted or precursor RNAs in which one of the nucleotides is modified to produce the correct sequence. Gott et al. (6) have observed four sites in the coI mRNA in which cytidines are converted to uridines. The presence of this activity in Physarum mitochondria lends credibility to the idea that the CU could be produced by nucleotide conversion.

The insertion of all four nucleotides to produce functional mRNAs, rRNAs, and tRNAs in mitochondria of Physarum sets this editing system apart from all others. This complexity could result from multiple mechanisms of concurrent nucleotide insertion or a single mechanism able to identify an editing site and insert the correct number and type of nucleotide at that position. The cyt b mRNA with the most noncytidine insertions of any Physarum RNA characterized to date exemplifies the complexity of this process. Further studies of partially edited cyt b mRNAs and comparison of the editing of cyt b mRNAs from other organisms should give insights into the mechanism of this unique type of RNA editing.

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