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RNA Editing Intermediates of *cox2* Transcripts in Maize Mitochondria

AUSTIN J. YANG AND R. MICHAEL MULLIGAN*

Department of Developmental and Cell Biology, University of California, Irvine, California 92717

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Eighteen cytidines are changed to uridines in the coding sequence of transcripts for cytochrome *c* oxidase subunit 2 (*cox2*) in maize mitochondria. The temporal relationship of editing and splicing was examined in *cox2* transcripts by sequence analysis of spliced and unspliced cDNAs. Cloned cDNAs of unspliced *cox2* transcripts ranged from clones with no edited nucleotides to completely edited forms, while spliced cDNAs were nearly completely edited. Incompletely edited transcripts in the nascent pool of unspliced transcripts represent intermediates of the editing process. These results indicate that editing proceeds without a strong directional bias and suggest that RNA editing is a posttranscriptional process.

RNA editing has been reported in the mitochondria of several organisms (1, 5, 6), including higher plant species (3, 9, 11). The nucleotide modifications observed in plant mitochondrial transcripts are usually C-to-U transitions and occasionally U-to-C changes (3, 9–11, 16). RNA editing occurs frequently in the coding sequence and usually results in a radical change of the amino acid specified by the codon that restores the evolutionarily conserved sequence of the polypeptide (3, 9, 11).

The RNA sequences of *cox2* transcripts were determined by direct RNA sequence analysis from maize polysomal RNA (Fig. 1) and is in accord with RNA sequence analysis recently reported for maize mitochondrial *cox2* transcripts (4). Total polysomal RNA was obtained from 7-day-old

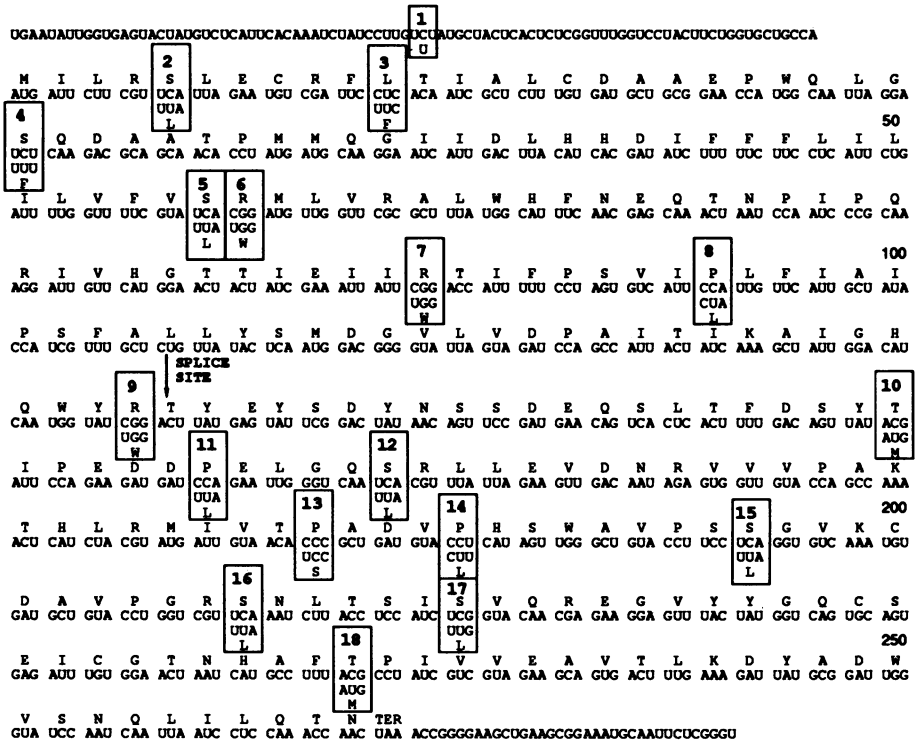

FIG. 1. RNA editing sites in *cox2* mRNA. The unedited RNA sequence for *cox2* is shown on the upper line (7). The edited RNA sequence was determined by direct RNA sequence analysis of polysomal RNA, and edited nucleotides and amino acid changes are shown beneath the unedited sequences. The RNA editing sites and the corresponding codons are identified numerically and with a box. Codon numbers are indicated above codons on the right.

* Corresponding author.

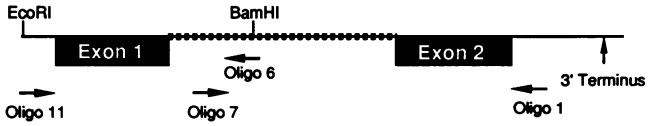

FIG. 2. Cloning of spliced and unspliced exons of *cox2* transcripts. The *cox2* gene has two exons and a single intron. The 5′ terminus of the transcript extends upstream of the *Eco*RI site, and the 3′ terminus of the transcript is shown by the vertical arrow. Unspliced cDNAs were amplified by PCR with oligonucleotides 7 and 1 or oligonucleotides 11 and 6. Spliced cDNAs were amplified by PCR with oligonucleotides 11 and 1. Oligonucleotides represent the following nucleotide sequences (nucleotide numbers of reference 7): 1, 1641 to 1660 (specific for 3′ untranslated flank); 6, 796 to 817 (intron specific); 7, 620 to 639 (intron specific); and 11, −68 to −45 (specific for 5′ untranslated flank).

etiolated maize seedlings (B37N) as previously described (12) and modified with the addition of 0.2% Triton X-100, 100 μg of chloramphenicol per ml, 0.5 mg of heparin per ml, and 0.4 M KCl to the homogenization medium. RNA sequence analysis was performed by dideoxy-chain termination of a cDNA synthesis reaction with ^{32}P-labeled synthetic oligonucleotides and avian myeloblastosis virus reverse transcriptase (8). Eighteen nucleotides in the coding sequence and one RNA edit site in the untranslated 5′-flanking region differ from the genomic DNA sequence (7); all sequence changes reflect a C-to-U transition. The 18 edited nucleotides in the coding sequence result in 17 changes in the amino acid sequence; edit site 11 has two edited nucleotides. Three additional edit sites in maize *cox2* transcripts were previously reported as partially edited sites; none of the partially edited sites modify the amino acid sequence (4). Direct RNA sequence analysis did not indicate partial editing at these sites; however, 1 of 15 spliced cDNA clones was edited at one of these sites (codon 10, nucleotide 30). Several additional cases of editing were infrequently observed by sequence analysis of cDNA clones and were not detected by direct RNA sequence analysis (data not shown).

Primary transcripts for *cox2* are processed to the mature form by splicing of a single intron (7) (Fig. 2). The temporal relationship between RNA editing and intron splicing was examined by sequence analysis of cDNA clones of unspliced *cox2* transcripts. Unspliced cDNAs were obtained by reverse transcription of total mitochondrial RNA with oligonucleotide 1 and polymerase chain reaction (PCR) amplification (15) of unspliced exon 1 or 2 with oligonucleotides 6 and 11 or 7 and 1, respectively (Fig. 2). PCR products were cloned into the Bluescript vector.

Sequence analysis of 10 cDNA clones for each unspliced exon indicated that a spectrum of completely unedited to completely edited cDNA clones was present (Fig. 3). The genomic DNA sequence, a completely edited cDNA, and two incompletely edited cDNAs are shown in Fig. 4. The cDNA clone in Fig. 4C is processed at edit sites 10 and 11 but not at site 12; the clone in Fig. 4D is edited at site 10 and at only one of two nucleotides in site 11.

The presence of incompletely edited mRNAs in the transient population of unspliced transcripts suggests that these transcripts represent intermediates in the RNA editing process (Fig. 3). Incompletely edited transcripts have been previously observed in *Oenothera* mitochondria, although it was uncertain whether the incompletely edited transcripts represented intermediates or an inefficiency in RNA editing (17). The cDNA clones for unspliced *cox2* transcripts represent transcripts in different stages of editing (Fig. 3). The modified sites in transcripts with very few edited sites (clones 2 to 5; clones 12 to 14) suggest that the first RNA editing events may occur at any of several editing sites. The modifications in transcripts with an intermediate number of edited sites (clones 5 to 7 and clones 14 to 16) indicate that editing tended to occur in clusters that might reflect RNA editing domains. No strong directional bias is indicated by these RNA editing intermediates from maize mitochondria, in contrast to the 3′-to-5′ polarity of the editing process in kinetoplast transcripts from parasitic protozoa (2, 18).

The relative abundance of incompletely edited RNAs in the nascent population of unspliced *cox2* transcripts suggests that RNA editing is a posttranscriptional process. The data in Fig. 3 indicate that only 61% (55 of 90) and 73% (66 of 90) of the edit sites were modified in the 10 unspliced cDNA clones for exons 1 and 2, respectively. In contrast, 99% (267 of 270) of the edit sites were modified in the 15 spliced cDNA clones of *cox2* (see below). Thus, a temporal correlation exists: the nascent population of transcripts is incompletely processed with respect to both splicing and editing, but the mature population of transcripts is both spliced and virtually homogeneously edited. In addition, more than half of the cDNA clones for exon 1 were derived from incompletely edited transcripts (Fig. 3), although the cDNA synthesis was primed in the 3′ untranslated region (Fig. 2). Thus, transcription of these unedited mRNAs had proceeded more than

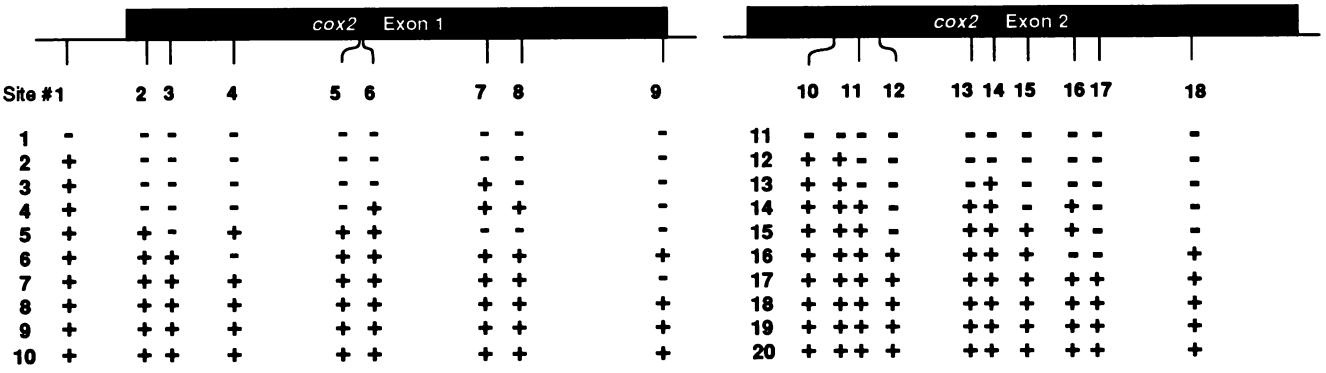

FIG. 3. Summary of RNA editing sites in exons of unspliced *cox2* transcripts. cDNAs of unspliced *cox2* transcripts were prepared from total mitochondrial RNA (14), amplified by PCR, and cloned into Bluescript. Dideoxy sequence analysis of cDNA clones was performed to evaluate the degree of editing. Edited or unedited nucleotides are indicated by + or −, respectively.

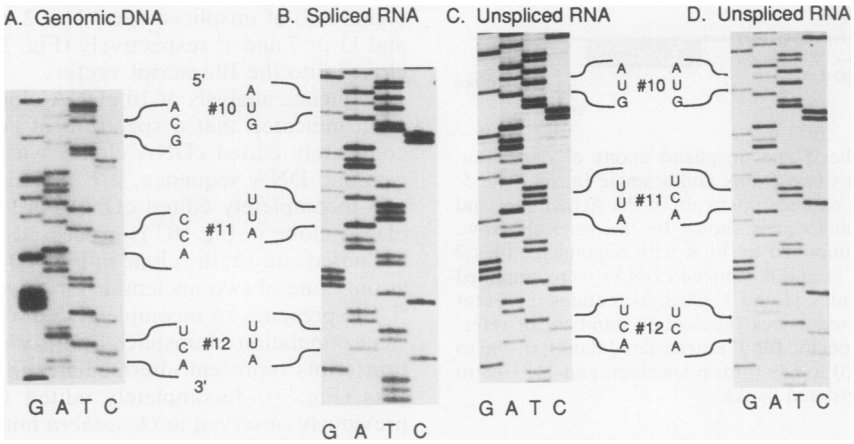

FIG. 4. Exon sequences in RNA editing intermediates cloned from unspliced *cox2* transcripts. Genomic and cDNA clones of *cox2* were sequenced by chain termination with Sequence (U.S. Biochemical). (A) DNA sequence from a genomic clone; (B) DNA sequence from a completely edited and spliced cDNA; (C and D) DNA sequence from two incompletely edited and unspliced cDNAs.

1,600 nucleotides beyond edit site 2, but the RNA sequence in the 5′ region had not been completely edited. These data indicate that RNA editing is not cotranscriptional (completed simultaneously with nucleotide insertion), and a given region of the transcript may remain unedited substantially after polymerization by RNA polymerase. Although it is not possible to demonstrate a precursor-product relationship among the RNA editing intermediates and mature mRNAs by these experiments, the results are consistent with a posttranscriptional mechanism of RNA editing.

Completely edited, unspliced cDNA clones were represented for each exon of *cox2*. Thus, splicing occurs somewhat more slowly than editing, and RNA editing may normally precede splicing in the processing of *cox2* transcripts. However, incompletely edited cDNAs for spliced transcripts of *cox2* are present in maize mitochondria (see below), and complete editing is not a strict prerequisite for splicing. Edit site 9 is within intron binding sequence 1 of the group II intron of *cox2* (13); it is not known whether editing at this site affects splicing.

The extent of RNA editing in spliced *cox2* transcripts from the polysomal RNA fraction was also examined. Direct RNA sequence analyses with polysomal RNA suggested that the RNA was homogeneous, with no indication of polymorphism in the population of transcripts (summarized in Fig. 1), although low-abundance forms would not be detected by this analysis. To further examine the extent of RNA editing of spliced *cox2* transcripts, cDNA clones were obtained from total maize polysomal RNA (Fig. 2). DNA sequence analysis of 15 cDNA clones indicated that most of the spliced *cox2* transcripts in the polysomal RNA fraction were completely edited. Twelve cDNA clones were completely edited; however, three spliced cDNA clones were unedited at a single site (edit sites 3, 12, and 17; data not shown). If the cDNA clones were derived from transcripts that were engaged in translation, and not from nonpolysomal contamination, the incompletely edited transcripts at sites 12 and 17 would code for the synthesis of polypeptides with radical amino acid substitutions (Fig. 1). The presence of incompletely edited polysomal transcripts raises the possibility that expression of *cox2* may be polymorphic in maize mitochondria.

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