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NOTES

Editing of Pre-mRNAs Can Occur before *cis*- and *trans*-Splicing in *Petunia* Mitochondria

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Plant mitochondrial mRNAs have recently been shown to undergo editing, involving cytidine-to-uridine changes relative to the DNA sequence. We have examined the temporal relationship of editing and intron removal in *coxII* mRNAs in *Petunia* mitochondria. By using differential hybridization to probes specific for edited and unedited RNA and by sequencing of individual unspliced *coxII* pre-mRNA cDNAs, we found that RNA editing at any editing site can precede the splicing event. Similar results were obtained from examinations of pre-mRNA cDNAs of *nad1*, a gene composed of multiple exons that are both *cis* and *trans* spliced. Thus, intron removal is not required before editing can occur. The existence of editing intermediates indicates that the editing process is not strictly coincident with transcription.

The mRNAs of plant mitochondria have recently been shown to be modified; at specific locations, uridine residues are found where cytidine is predicted from the genomic sequence (6, 12, 15). RNA editing in which C is changed to U has also been observed in the human apolipoprotein B mRNA (16). However, editing in the apolipoprotein B mRNA occurs only at one site, while in plant mitochondria, as many as 10 C's in a 75-codon mRNA can be edited to U's (23). Even so, RNA editing in plant mitochondria is less extreme than that found in trypanosome species, in which multiple U residues are inserted at specific points in kinetoplast mRNAs (1, 9, 20, 21).

Little information is available on the mechanism of RNA editing in plant mitochondria. In trypanosomes, guide RNAs (gRNAs) have been postulated to provide the information necessary for U insertion. Trypanosome transcript analysis has led to a hypothesis that the editing process occurs in a 3′-to-5′ direction along the initial transcript (3, 8, 22). So far, no gRNAs have been detected in plant mitochondria (13). There are no obvious sequence similarities in the regions flanking the edited C's other than a high probability of a pyrimidine and the absence of G immediately preceding the edit site (7). Plant cDNAs have been found which exhibit editing in some but not all potential editing sites, with no obvious polarity (19). Such cDNAs have been interpreted as editing intermediates, although there is no evidence that the corresponding transcripts are further processed.

We were interested in determining the relative timing of RNA editing versus transcription and intron excision. The *coxII* gene, encoding subunit two of cytochrome oxidase, contains an intron in several plant species (7, 17). Edited sites have been found in spliced *coxII* mRNAs in wheat, pea, and maize (7) and are reported here in *Petunia hybrida*. In *P. hybrida*, there are two mitochondrial *coxII* genes, named *coxII-1* and *coxII-2*, which contain group II introns of 1,353 and 1,354 nucleotides, respectively (17). The DNA sequence of exon 1 of the two genes is identical, while the exon 2 sequences are identical until 12 bases before the *coxII-1* stop codon (17; Fig. 1). The *coxII-2* coding region is 48 codons longer than that of *coxII-1*. The *Petunia coxII* gene coding regions are adjacent to a larger repeated segment of the mitochondrial genome (10). Both *coxII* genes yield multiple transcripts arising within the larger repeat; three 5′ termini and two 3′ termini have been detected (17; Fig. 1).

The edited sites found in the *Petunia* mature *coxII* mRNA were determined by comparing the sequences of cDNA clones from a mitochondrial cDNA library and clones of cDNA amplified by the polymerase chain reaction (PCR) with the previously determined genomic sequence (17). The mitochondrial cDNA library was constructed by using random hexanucleotide primers. cDNAs were cloned into λ-ZAPII (Stratagene), using *Not*I-*Eco*RI adaptors. *coxII*-specific cDNAs were isolated by hybridization with an exon 1-specific probe. Positive clones were converted into pBluescript plasmids by in vivo excision and were subjected to double-stranded sequencing. Amplification of spliced products representing *coxII-2* was accomplished by PCR using a *coxII-2*-specific 3′ primer (primer 2; Fig. 1) and a 5′ primer from exon 1 (primer 1; Fig. 1); the 900-bp PCR product was cloned into Bluescript (Stratagene) and sequenced. Fifteen clones were sequenced from products of a single amplification of total flower bud RNA; seven clones were sequenced from products of two amplifications of mitochondrial RNA from suspension cells; tissue-specific editing was not detected. The oligonucleotides used for amplification were CG TATCATGGATCCTGGGTC (primer 1) and CTTGAGAC GAGCTGCGTCTG (primer 2). The underlined C is a substitution to create a *Bam*HI site.

A schematic of the sites where editing can occur is shown in Fig. 1. There are seven edit sites in the portion of *coxII* mRNA derived from exon 1 and seven edit sites in the exon 2 region. A fully edited transcript would thus have 14 C-to-U changes relative to the genomic DNA sequence. Most cDNAs sequenced were fully edited (see below). All edited codons specify an amino acid different from those predicted

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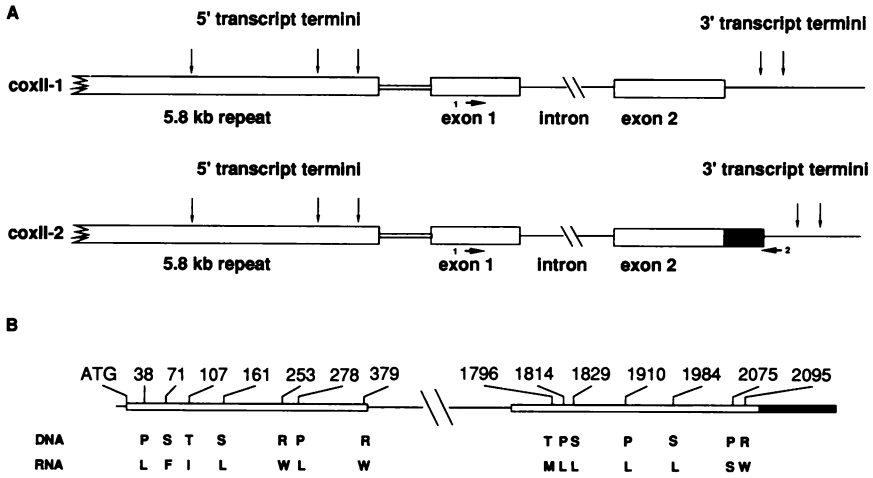

FIG. 1. (A) Diagram of *coxII* genes and transcripts in *P. hybrida*. Exons 1 and 2 are indicated; the *coxII-2* coding region extension (17) is stippled. Upstream of both *coxII-1* and *coxII-2* is a 5.8-kb sequence which is repeated three times in the mitochondrial genome (10). Vertical arrows denote the 5′ and 3′ transcript termini according to S1 nuclease protection experiments (17). Horizontal arrows denote locations of primer targets for PCR amplification. (B) Schematic diagram of the edited sites found in *coxII* transcripts. Edited nucleotides are numbered from the beginning of the coding region (A of ATG = 1), including the intervening sequence of the intron, using the *coxII-1* sequence (17) for numbering. The amino acids predicted by the genomic sequence and the edited sequence are shown below the edit sites.

from the genomic sequence. No editing sites were found in the divergent 3′ region of *coxII-2*.

To examine editing in the unspliced and spliced mRNA populations, we first analyzed *coxII* transcripts on RNA blots. We probed blots of *Petunia* mitochondrial RNA with oligonucleotides that would hybridize preferentially to edited or unedited transcripts. Two of the exon 2 edits were within 18 nucleotides of each other (at nucleotides 1796 and 1814); these were incorporated into a 25-nucleotide edited oligonucleotide complementary to the edited RNA. An unedited oligonucleotide was synthesized that was complementary to the RNA predicted from the genomic DNA sequence (Fig. 2).

Control sense and antisense RNAs were transcribed from genomic (unedited) and edited exon 2 sequences subcloned in Bluescript. Those RNAs and 20 μg of mitochondrial RNA from *P. hybrida* 3704 suspension cells were electrophoresed in duplicate on 1.1% agarose (Seachem HGT)-formaldehyde gels and subsequently blotted to nitrocellulose. The filters were prehybridized for 3 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2× Denhardt's solution–50 μg of salmon sperm DNA per ml–50 μg of *Escherichia coli* tRNA per ml–0.1% sodium dodecyl sulfate (SDS) at 42°C. Filters were hybridized with 20 ng of the probe oligonucleotide labeled by T4 polynucleotide kinase with [γ-^{32}P]ATP and 200 ng of unlabeled competitor oligonucleotide (e.g., edited oligonucleotide competed with unedited probe oligonucleotide) in the same buffer overnight at 42°C. Filters were then washed twice at room temperature in 2× SSC–0.5% SDS and then twice at 60°C in 6× SSC–0.1% SDS for 10 min prior to exposure of the film.

The oligonucleotide complementary to the edited RNA efficiently hybridized to several transcripts (Fig. 3B, lane 3), while no hybridization was detected to a control lane containing unedited RNA transcribed in vitro (Fig. 3A, lane 1). The largest band detected by hybridization with the edit-specific probe was also detected with an intron-specific

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--AUACGAUUCCAGAAGAUGAUCCAGA--
  TATGCTAAGGTCTTCTACTAGGTCT
        UNEDITED
     OLIGONUCLEOTIDE

--AUAUGAUUCCAGAAGAUGAUCUAGA--
  TATACTAAGGTCTTCTACTAGATCT
        EDITED
     OLIGONUCLEOTIDE
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FIG. 2. Sequences of the two oligonucleotides complementary to edited and unedited RNA (bottom lines) and the corresponding portion of the *coxII* RNAs (top). The *coxII* mRNA sequence is shown 5′ to 3′, and the sequences of the oligonucleotides are shown in reverse orientation. The edit sites (C or U) are shown in bold type in each RNA segment.

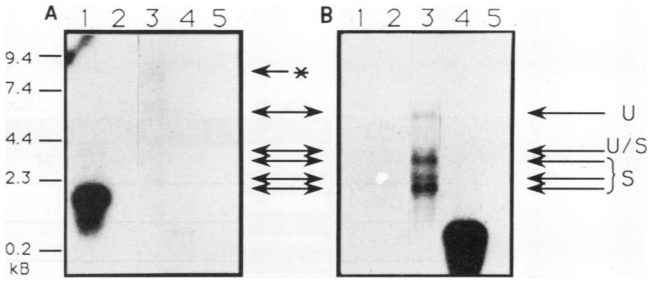

FIG. 3. RNA blot hybridization of oligonucleotides to mitochondrial RNAs and control mRNAs produced in vitro. Control RNAs were transcribed by using either T7 (lanes 1 and 4) or T3 (lanes 2 and 5) RNA polymerase from genomic (lanes 1 and 2) and edited (lanes 4 and 5) exon 2 sequences. Lane 3 contained 20 μg of *P. hybrida* mitochondrial RNA. Electrophoresis, hybridization, and washing were as described in the text. (A) Hybridization with the unedited oligonucleotide; (B) hybridization with the edited oligonucleotide. U represents locations of hybridization signals obtained with an intron-specific probe (not shown). By size, the shorter of these species could also represent spliced (S) transcript with one of the longer 5′ ends (see Fig. 1A). The band marked with * results from hybridization to DNA.

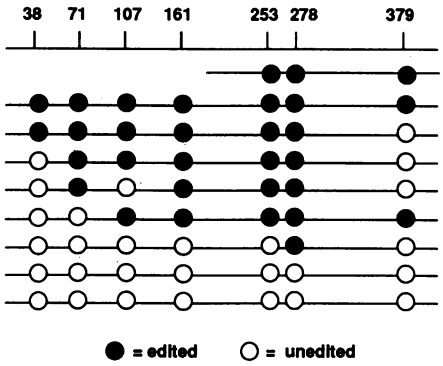

FIG. 4. Edits found in exon 1 of unspliced *coxII* cDNAs. Filled circles represent T's and open circles represent C's found at the edit sites in exon 1. All cDNAs sequenced extend 3′ beyond the exon 1 region. All but one extend 5′ beyond exon 1 as well.

probe (data not shown). No signal at this mobility was observed following probing with the unedited oligonucleotide. The oligonucleotide complementary to the unedited RNA did not detect *coxII* transcripts under conditions in which a strong signal resulted from hybridization to unedited RNA produced in vitro (Fig. 3A, lane 3). These observations led us to conclude that most of the unspliced as well as the spliced *coxII* mRNA is edited at one or both of these sites.

Further confirmation that editing can occur before splicing of *coxII* was obtained by examining unspliced RNA intermediates. We isolated a series of cDNA clones that hybridized to a *coxII* intron probe; these should represent pre-mRNAs that have not yet been spliced. We sequenced the exon 1 region of these clones, since RNA blot hybridizations had already indicated that positions 1796 and 1814 of exon 2 are edited in unspliced transcripts. The degree of editing of the unspliced *coxII* cDNAs is shown in Fig. 4. Most such clones represent transcripts which do not carry all possible edits, in contrast to the results obtained by sequencing of spliced cDNAs. Each potential edit site within the first exon is edited in at least one intron-containing cDNA. These cDNAs are likely to represent individual transcripts in which editing has not begun or been completed. No polarity of the editing process (3′ to 5′ or vice versa) is apparent from comparisons of partially edited RNAs with fully edited transcripts. No editing within the intron has been detected (data not shown).

In addition to the editing of *coxII* pre-mRNAs which will undergo *cis* splicing, pre-mRNAs of *nad1* (subunit 1 of the NADH dehydrogenase complex) are edited before *trans* splicing. The *Petunia nad1* gene is composed of five exons which must be joined by three *trans*-splicing and one *cis*-splicing event (data not shown; 5). cDNAs representing various pre-mRNA intermediates containing one or more exons were selected from cDNA libraries or obtained by PCR amplification of cDNA. The RNA editing sites in *nad1* were deduced from the cDNAs shown in Fig. 5 and from a PCR product which contains spliced exons b, c, d, and e. As was observed in the *coxII* cDNA clones, the mRNA intermediates are not fully edited compared with spliced transcripts, and there is no obvious polarity of editing (Fig. 5).

Our finding that editing of *coxII* mRNA at position 379, which is only 5 nucleotides upstream of the intron (16, 17), can occur before splicing, provides some information concerning sequence requirements for editing. Comparison of the edited sequences of mature *coxII* mRNA from wheat, maize, pea, *Oenothera* and *Petunia* shows that the corresponding codon is edited and that the RNA sequence is identical for 25 nucleotides 5′ to the edit site (4, 7, 11, 14, 17). However, *Oenothera* and pea *coxII* genes contain no intron. Thus, 3′ to the edit site, the sequence context of this edit site in non-intron-containing *Oenothera* and pea *coxII* transcripts is quite different from that of the intron-containing *Petunia coxII* transcripts. This suggests that any contextual information which may be required as a signal for editing of this site must reside upstream of the intron. Alternatively, if gRNAs exist in plant mitochondria, the gRNA for this site in the intron-containing species must be different than that in the non-intron-containing species.

According to the RNA blot analysis shown in Fig. 3, most spliced *coxII* mRNAs are edited at the two sites in exon 2 that were analyzed. However, four cDNA clones of spliced *coxII* mRNAs obtained from two separate PCR amplifications were not edited at one site—position 379 (Fig. 1B; data not shown). The codon at this position encodes tryptophan in other plants as well as in *Neurospora*, yeast, mouse,

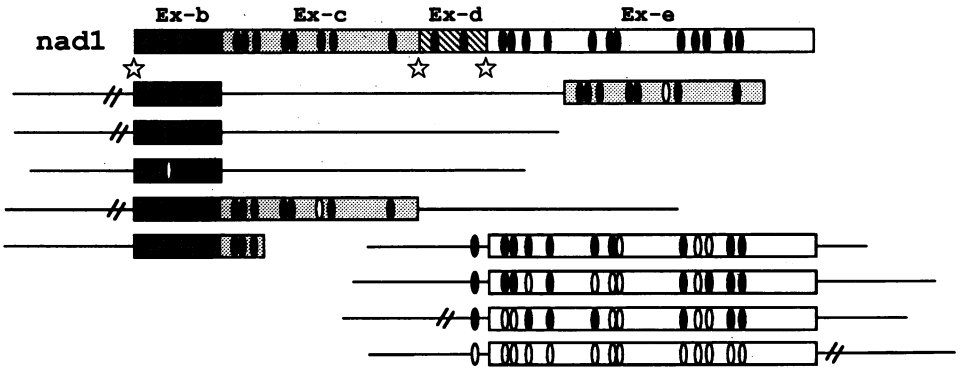

FIG. 5. Schematic of the RNA editing pattern in *Petunia nad1* mRNA. Shown on the first line is a representation of spliced *nad1* exons b, c, d, and e, with the sites of RNA editing shown as ovals. The stars denote the exon-exon junctions which are formed as a result of *trans* splicing. cDNAs were isolated which represent *nad1* RNA intermediates that contain unspliced and spliced exons b and c and unspliced exon e. The RNA editing sites which were edited in these cDNAs are shown as filled ovals; sites which have not undergone RNA editing are shown as open ovals. The flanking regions of these cDNAs are not shown to scale.

Xenopus, and *Drosophila* cells (2, 4, 11, 14). It is therefore unlikely that the protein product resulting from translation of a transcript lacking an edit at this site would produce a functional protein. Since unedited as well as edited transcripts of *coxII* contain the AUG start codon, it remains to be determined whether ribosomes can distinguish mRNAs that are fully edited from partially edited transcripts before initiating or completing translation.

The nucleotide sequences reported have been assigned GenBank accession numbers X17394 and X17395.

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