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RNA editing of ATPase subunit 9 transcripts in *Oenothera* mitochondria

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The mRNA for subunit 9 of the ATPase (atp9) in the higher plant *Oenothera* is edited in four nucleotide positions. Three events alter genomic serine and proline codons to triplets specifying leucine. A UGA termination codon is introduced into the reading frame by modification of a CGA arginine codon. This modification shortens the polypeptide by four amino acids. Direct sequencing of PCR amplified cDNA from the total mitochondrial mRNA population gives no indication of partially edited transcripts suggesting a rapid and efficient modification of atp9 transcripts in *Oenothera* mitochondria.

RNA editing; *Oenothera* mitochondria; ATPase subunit 9

1. INTRODUCTION

RNA editing in higher plant mitochondria alters the mRNA sequence to specify a polypeptide with a sequence different from the protein predicted by the gene [1-3]. In the plant species so far investigated in this respect, cytidines are substituted or altered to uridines. Only one exception has so far been observed for the reverse sequence alteration, a change from uridine to cytidine in the mRNA of the cytochrome *b* gene in *Oenothera* [4].

RNA editing has so far been found in mRNAs of all coding regions for mitochondrial proteins investigated in a number of species of higher plants, for example, in wheat [2,3], pea [5], *Oenothera* [1,6,7] and carrot (B. Wissinger, unpublished observations).

The edited mRNA sequence does appear to encode the actually synthesized polypeptide at least in the one case where protein sequence data are available for a plant mitochondrial protein specified by the organellar genoma [8]. The partial amino acid sequence determined for wheat subunit 9 of the ATPase (ATP9) differs from the polypeptide predicted by the DNA sequence and can be derived by several singular RNA editing events in the mRNA.

We have now analyzed the extent of RNA editing in the *Oenothera* mRNA coding for this polypeptide and find four sites edited within the coding region, the last of which creates a new termination codon and shortens the encoded protein.

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2. MATERIALS AND METHODS

2.1. *Isolation of mitochondrial RNA*

Mitochondrial RNA (mRNA) was prepared from *Oenothera berteriana* tissue cultures and purified by pelleting in a CsCl gradient as described previously [9].

2.2. *cDNA synthesis*

ATPase subunit 9 cDNA was prepared by reverse transcription from a 16 nucleotide primer (5'-GCTTGCTTTATGAGCC-3') complementary to a sequence 3' of the atp9 open reading frame [10]. The reaction contained 10 μg total mtRNA (incubated for 1 min at 95°C and quick-cooled) in 50 mM Tris (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$ 1 mM DTT, 1.25 mM each of dATP, dGTP, dTTP and dCTP, 10 U RNasin, 0.5 μg of the 3'-primer and 10 U AMV-reverse transcriptase (Boehringer Mannheim). The reaction was incubated for 1 h at 37°C and terminated by heat for 3 min at 95°C. 30 μl of 0.7 M NaOH and 40 mM EDTA were added to the 20 μl first strand cDNA synthesis reaction and incubated at 65°C for 10 min to remove the RNA. The single-stranded cDNA was precipitated and washed with 80% Ethanol.

2.3. *Amplification and sequencing of the cDNA*

Half of the cDNA thus obtained was amplified by the PCR between the 16mer 3'-primer and an 18mer 5'-primer with the sequence 5'-GAACGGGAACAATTGATC-3' complementary to the cDNA upstream of the open reading frame. The 100 μl PCR mixture contained 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris (pH 8.5 at 37°C), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.5 μg of each primer and 3 U Taq polymerase (Boehringer Mannheim). The first cy-

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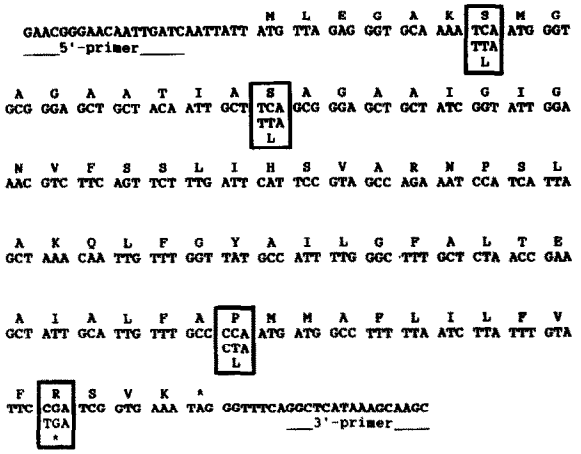


Fig. 1. Nucleotide sequence of the open reading frame encoding subunit 9 of the ATPase in *Oenothera* mitochondria. Sequences of genomic and cDNAs were analysed between the two primers indicated by the thin lines. The amino acid sequence of the genomic reading frame is given above the triplets in the single letter code. For the cDNA sequence only codons altered by RNA editing are shown and translated underneath (boxes).

cle included 3 min at 100°C prior to the addition of the polymerase, 2 min at 37°C during which the Taq polymerase was added and 4 min at 72°C. This cycle was followed by 40 cycles of 1 min at 94°C, 2 min at 37°C and 2 min at 72°C which were extended by 5 s each cycle.

Products were analysed on an agarose gel after the PCR. The amplified fragment was cut out and electroeluted. Fragment ends were polished with T4-DNA polymerase, phosphatased and ligated into pBluescript vectors. Single-stranded DNA was generated in an additional round of the PCR with asymmetric primer concentrations (100-fold reduction of one of the primers). Another 40 cycles of PCR generated enough single-stranded DNA for direct sequence analysis. Free nucleotides and primers were removed by passage through a Qiagen tip-5 column. Sequencing reactions from the appropriate primers were done with a T7-sequencing kit (Pharmacia).

Compression in the sequencing gel around nucleotide 50 had led to misinterpretation of the sequence [9] that can now be corrected (Fig. 1).

3. RESULTS

Sequence analysis of cDNAs from primers flanking the atp9 coding region shows four nucleotide differences to the genomic sequence (Fig. 1). All four nucleotide alterations observed in this mRNA are cytidine to uridine modifications. Two of the amino acid alterations within the coding sequence change TCA serine codons to TTA leucine triplets. One proline CCA codon is modified to a leucine CTA triplet. The fourth alteration has not been observed previously in the other genes analysed so far in plant mitochondria and modifies a CGA arginine triplet to a TGA termination codon.

This termination codon shortens the deduced polypeptide length by four amino acids to the effect that the carboxy-terminus of the *Oenothera* atp9 polypeptide now conforms with the length of the maize, pea and yeast polypeptides (Fig. 2). The three amino acid alterations within the coding regions introduce leucine instead of the genomic serine and proline residues. The first leucine at amino acid position seven is not conserved in the different species for which the polypeptide sequences are aligned in Fig. 2, whereas both leucines at positions 17 and 64 appear to be well conserved in evolution and are presumably important for the function of this ATPase subunit.

Efficiency of RNA editing process in the atp9 coding region was investigated by direct sequence analysis of the complete PCR amplified atp9-specific cDNA population. This sequence reflects the in vivo distribution of nucleotides edited in the mRNA molecules encoding this polypeptide. Analysis of the PRC-products by sequencing indicates that all four editing sites are altered efficiently in the RNA (Fig. 3). Long exposures of these gels show a complete shift of the genomic C to T in the cDNA at all edited positions (Fig. 3b).

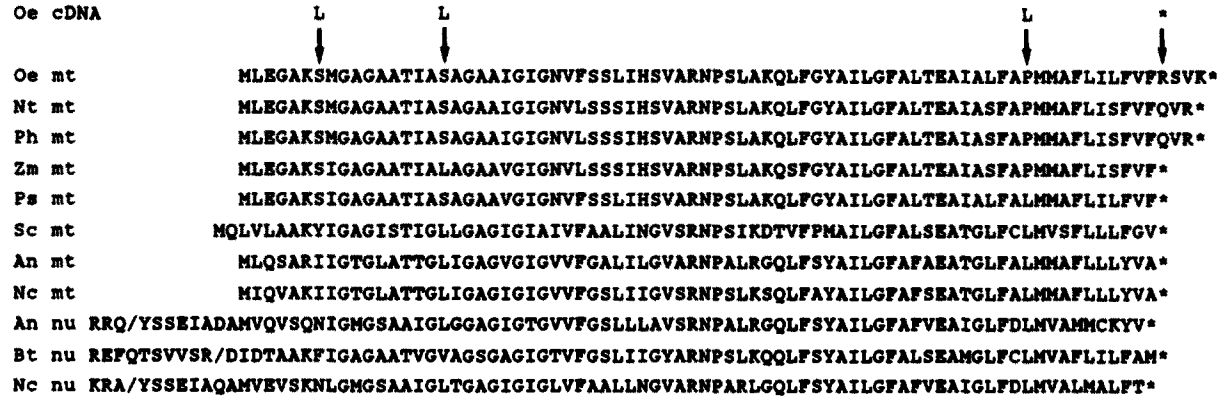


Fig. 2. Amino acid comparison between the ATPase subunit 9 polypeptides specified by the cDNA sequence in *Oenothera* (Oe cDNA) and the genomic sequences in mitochondria of *Oenothera* (Oe mt), the four plant mitochondrial genomic sequences from *Nicotiana tabacum* (Nt mt) [10], *Petunia hybrida* (Ph mt) [11], *Zea mays* (Zm mt) [12] and *Pisum sativum* (Ps mt) [13], the three fungal mitochondrial genomic sequences from *Saccharomyces cerevisiae* (Sc mt) [14], *Aspergillus nidulans* (An mt) [15] and *Neurospora crassa* (Nc mt) [15] and three nuclear encoded genes from *Aspergillus nidulans* (An nu) [16], *Bos taurus* (Bt nu) [17] and *Neurospora crassa* (Nc nu) [18]. Only the amino acids altered by RNA editing and the mRNA encoded termination codon are shown for the *Oenothera* cDNA sequence. Details of the comparison are given in the text.

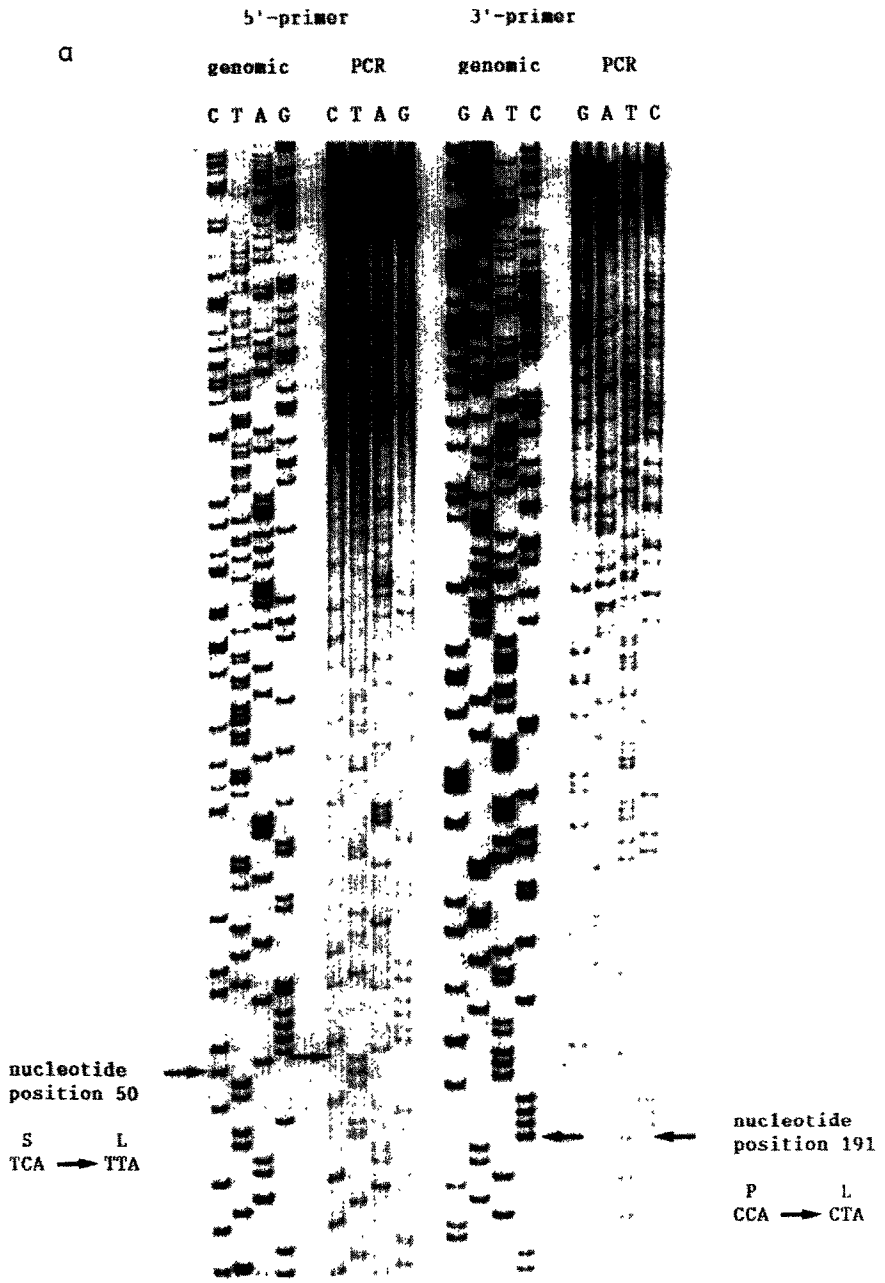
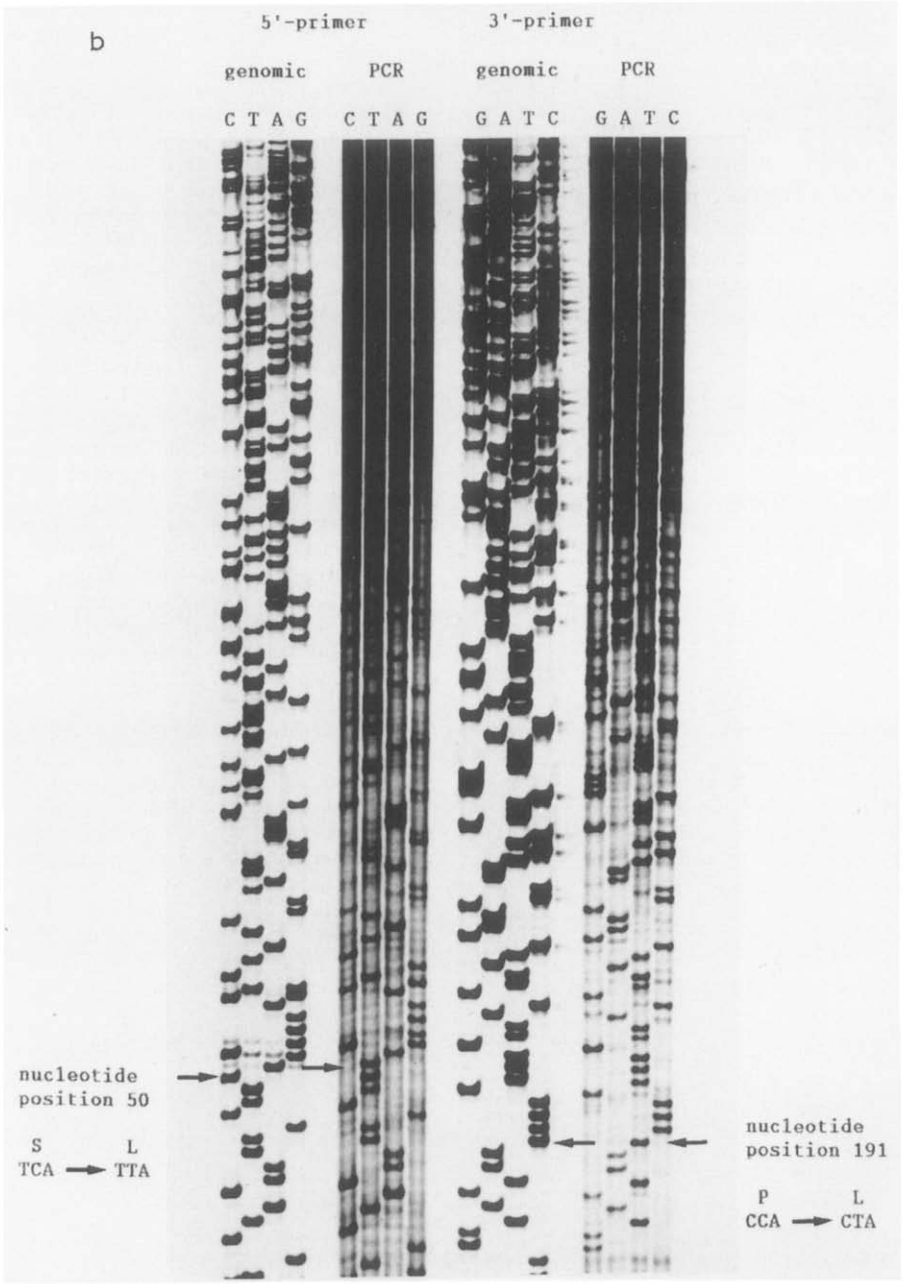


Fig. 3. Sample gel of the nucleotide sequence determination of genomic and PCR-amplified cDNA fragments (PCR). Two sites edited in the cDNA sequence are indicated. Nucleotide numbering starts at the first nucleotide of the initiation codon. Sequencing reactions are shown from both 3'- and 5'-primers as indicated. The altered triplets are given with the genomic triplet on the left and the edited codon on the right with the

4. DISCUSSION

The high degree of conservation in length and composition of the ATPase subunit 9 between different species indicates strong functional constraints acting on this polypeptide. RNA editing of the atp9 mRNA in plant mitochondria further supports the evolutionary stability of this polypeptide.

The atp9 protein encoded by the *Oenothera* mRNA is much better conserved in evolutionary terms after introduction of the termination codon and the three amino acid alterations than for example mitochondrially encoded subunits of the NADH-dehydrogenase. The limited variability of the atp9 polypeptide sequence appears to have consequences also for the RNA editing process in plant mitochondria. Editing of the mRNA sequence must for example proceed very efficiently in this region to ensure complete editing at all positions before the mRNA is made accessible to the ribosome. Translation of partially edited transcripts would lead to ifferent polypeptides that might be disatrous to the function of the ATPase complex. Indeed the entire



respective amino acid in the single letter code identified above. (a) The short exposure of this gel allows the identification of the nucleotide alterations. The compression above (3'-) of nucleotide position 50 is explained in the text. (b) The long exposure of this gel exemplifies the efficiency of the RNA editing process in this gene which has converted virtually 100% of all transcripts.

mitochondrial atp9 mRNA population in *Oenothera* seems to be edited at all sites (Fig. 3).

Differentially edited mRNAs have been found for subunit 3 of the NADH dehydrogenase (nad3) in *Oenothera* mitochondria [6]. This polypeptide appears to be less conserved in evolution and might tolerate slightly divergent polypeptides without significant loss of function. The protein variants specified by the differentially edited nad3 mRNAs in *Oenothera* mitochondria [6] might thus be able to perform the required functions.

The high degree of conservation of the ATPase subunit 9 on the other hand may even allow the prediction of potential editing sites in other plant species. The nucleotides edited in *Oenothera* will thus also be altered in the other plants unless the amino acid specified by the edited mRNA is already encoded by the genomic sequence like amino acid 17 in maize and amino acid 64 in pea (Fig. 2).

RNA editing is likewise predicted to alter the CAA at amino acid position 75 to a TAA termination codon in tobacco and *Petunia* to generate the same carboxy-

terminus in these species as in *Oenothera*, maize and pea. As further examples amino acids 31, 61 and 71 are likely to be edited in tobacco, *Petunia* and maize from serine to leucine codons.

This analysis suggests that atp9 mRNAs will be edited in all of the plant species aligned here. Up to data no experimental data have been reported of RNA editing in mitochondria from other plant species than *Oenothera*, wheat, pea and carrot. Comparisons like the above however strongly suggest RNA editing to be a general principle of higher plant mitochondria.

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REFERENCES

[1] Hiesel, R., Wissinger, B., Schuster, W. and Brennicke, A. (1989) Science 246, 1632–1634.
[2] Gualberto, J.M., Lamattina, L., Bonnard, G., Weil, J.-H. and Grienenberger, J.-M. (1989) Nature 341, 660–662.
[3] Covello, P.S. and Gray, M.W. (1989) Nature 341, 662–666.
[4] Schuster, W., Hiesel, B., Wissinger, B. and Brennicke, A. (1990) Mol. Cell Biol. 10, 2428–2431.
[5] Covello, P.S. and Gray, M.W. (1990) in: Florida Winter Organelle Meeting (Cline, K., Hauswirth, W.W., Laipis, P., Lewin, A., O'Brien, T.W. and Pring, D.R. eds) p. 17, Clearwater, Fl.
[6] Schuster, W., Wissinger, B., Unseld, M. and Brennicke, A. (1990) EMBO J. 8, 263–269.
[7] Schuster, W., Unseld, M., Wissinger, B. and Brennicke, A. (1990) Nucleic Acids Res. 18, 229–233.
[8] Graves, P.V., Begu, D., Velours, J., Neau, E., Belloc, F., Litvak, S. and Araya, A. (1990) J. Mol. Biol. (in press).
[9] Schuster, W. and Brennicke, A. (1989) Curr. Genet. 14, 187–192.
[10] Bland, M.M., Levings III, C.S. and Matzinger, D.F. (1986) Mol. Gen. Genet. 204, 8–16.
[11] Rothenberg, M. and Hanson, M.R. (1987) Mol. Gen. Genet. 209, 21–27.
[12] Dewey, R.E., Schuster, A.M. Levings III, C.S. and Timothy, D.H. (1985) Proc. Natl. Acad. Sci. USA 82, 1015–1019.
[13] Morikami, A. and Nakamura, K. (1987) Nucleic Acids Res. 15, 4692.
[14] Hensgens, L.A.M., Grivell, L.A., Borst, P. and Bos, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 1663–1667.
[15] Brown, T.A., Ray, J.A., Waring, R.B., Scazzocchio, C. and Davis, R.W. (1984) Curr. Genet. 8, 489–492.
[16] van den Boogaart, P., Samallo, J., van Dijk, S. and Agsteribbe, E. (1982) in: Mitochondrial Genes (Slinimski, P., Borst, P. and Attardi, G. eds) pp. 375–380, Cold Spring Harbor Press, Cold Spring Harbor.
[17] Ward, M. and Turner, G. (1986) Mol. Gen. Genet. 205, 331–338.
[18] Sebald, W., Hoppe, J. and Wachter, E. (1979) in: Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E. ed.) pp. 63–74, Elsevier, Amsterdam.
[19] Sebald, W., Hoppe, J. and Wachter, E. (1980) Proc. Natl. Acad. Sci. USA 77, 785–789.