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RNA editing makes mistakes in plant mitochondria: editing loses sense in transcripts of a rps19 pseudogene and in creating stop codons in coxI and rps3 mRNAs of *Oenothera*

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ABSTRACT

An intact gene for the ribosomal protein S19 (rps19) is absent from *Oenothera* mitochondria. The conserved rps19 reading frame found in the mitochondrial genome is interrupted by a termination codon. This rps19 pseudogene is cotranscribed with the downstream rps3 gene and is edited on both sides of the translational stop. Editing, however, changes the amino acid sequence at positions that were well conserved before editing. Other strange editings create translational stops in open reading frames coding for functional proteins. In coxI and rps3 mRNAs CGA codons are edited to UGA stop codons only five and three codons, respectively, downstream to the initiation codon. These aberrant editings in essential open reading frames and in the rps19 pseudogene appear to have been shifted to these positions from other editing sites. These observations suggest a requirement for a continuous evolutionary constraint on the editing specificities in plant mitochondria.

INTRODUCTION

RNA editing in plant mitochondria alters codon identities in all open reading frames investigated in this respect (1, 2, 3). Most of the resulting codon exchanges improve the similarity of the specified polypeptide with the respective proteins of other species. Many of the arginine CGG codons for example are edited to tryptophan UGG codons where the latter amino acid is conserved in other species at this position and is presumably important for correct function of the protein. However, not all CGG codons are edited and not all editing sites result in amino acid alterations. Some of the editing events change genomic encoded cytidines to uridines in the mRNA sequences in silent codon positions. In these instances the degeneracy of the genetic code provides for the same amino acid to be specified by both edited and unedited codons.

RNA editing events in plant mitochondria have furthermore been observed in non-coding regions of mRNAs and in one cDNA clone also in the 26S rRNA (3, 4, 5, 6). The functional significance of most of these events is unclear. One editing event upstream of the rps14 open reading frame may improve a potential ribosome binding site (4), some editing sites in intron sequences improve base-pairings in the secondary structure model and may thus be required for correct processing of these introns (5).

The RNA editing events in non-coding regions show that the editing process is not connected with translated sequences and is uncoupled from the triplet code. To date no information is available on the nature of the specificity determinant(s) of RNA editing in plant mitochondria. The primary sequences surrounding editing sites show no overt similarity and do not appear to be solely responsible for conferring specificity to a cytidine to be edited (6, 7, 8). The RNA editing activity in plant mitochondria nevertheless can select with high specificity the nucleotides to be edited while leaving adjacent and nearby nucleotides unaltered. In all mRNA sequences investigated to date no obviously 'wrong' editing has been identified. This high specificity was equally observed in genes with very efficient editing sites like atp9 and in genes where editing sites are found processed in only part of the steady state mRNA population (9, 10).

Analysis of RNA editing in transcripts of the rps19 pseudogene of *Oenothera* mitochondria now reveals that RNA editing in this apparently useless sequence has drifted away from 'good' editings improving the protein similarity with other species to 'bad' editings that lessen the degree of conservation. This finding and the observation of low-frequency stop codons created by RNA editing in the coxI and rps3 genes in *Oenothera* show that the specificity of RNA editing in plant mitochondria is prone to shifting within an edited region.

MATERIALS AND METHODS

Mitochondrial nucleic acids were prepared from *Oenothera berteriana* tissue culture cells by procedures previously described (11). The clones used in this investigation were isolated from established genomic and cDNA libraries of *Oenothera* mitochondrial DNA and RNA (5, 11). Handling and analysis of nucleic acids in hybridisation, PCR and sequence determinations followed standard protocols (12). Primers for sequence analysis and PCR amplification of the rps19 coding region were 5'-ATACATATGCCACGACGATCTATATGG-3' and 5'-TCTAGAGGATCCTTAGACTTTACTTTTTCC-3'. The primer for analysis of the coxI clones was 5'-CTTGACGAAGTAAAGCTGTCTG-3'.

RESULTS

Analysis of the sequences surrounding a region with coxII and atpA sequence similarities in the *Oenothera* mitochondrial genome (13) revealed upstream of this region another sequence fragment with similarity to rps19 sequences of other species (Figure 1). Hybridisation experiments with this pseudogene sequence identified cDNA clones derived from another location in the mitochondrial genome. Here similarity extends over the full length of the rps19 open reading frame. This genomic sequence is located in a ribosomal protein gene cluster in *Oenothera* mitochondria including also the rps3 gene, previously identified in maize mitochondria (14). The gene order is conserved with the respective ribosomal protein gene cluster in *E. coli*. The rps19 and rps3 sequences are cotranscribed in *Oenothera* mitochondria, since cDNA clones were identified covering both gene sequences.

While this work was in progress a report of the rps19 gene in *Petunia* mitochondria was published (15), which appears to be located in a conserved position in the ribosomal protein cluster. The sequence downstream of this open reading frame in *Petunia* contains the first exon and part of the adjacent intron of the rps3 gene. An rps19 pseudogene sequence is present in the mitochondrial genome of a fertile rice line just downstream of the atp6 open reading frame (data not shown and reference 16).

A TGA codon and RNA editing in rps19 mRNAs

The genomic region encoding the rps19 sequence with similarity over the entire length to the respective bacterial and *Petunia* rps19 open reading frames (15) was analysed in both genomic and cDNA clones of *Oenothera* (Figures 1 and 3A). Two copies of this sequence are present in the mitochondrial genome of this higher plant (Figure 3A, B). Both locations were sequenced completely and found to contain the entire rps19 sequence 13 nucleotides upstream of the rps3 initiation codon (Figure 3C; 14). Sequences are identical up to the point of recombination in the rps3 intron sequence (14), which exchanges the downstream rps3 coding region with a different genomic sequence resulting in an intact copy of the rps3 gene and a second copy truncated in the rps3 intron sequence. The genomic arrangement with the truncated copy of the rps3 gene is much less abundant in the mitochondrial DNA than the intact copy (Figure 3B). The 'substoichiometric' abundance suggests some low copy number molecules of the complex mixture of DNA molecules in *Oenothera* mitochondria to contain this sequence (13, 17, 18).

Genomic sequences from cloned restriction fragments, PCR products of mitochondrial DNA and all cDNA clones of the rps19 homology in *Oenothera* mitochondria contain a TGA stop codon in the otherwise open reading frame (Figure 3C). The triplet corresponding to this TGA stop is encoded in *Petunia* mitochondria as a GGA codon specifying a highly conserved glycine residue (Figure 3D; 15). The amino acid sequences deduced from the rps19 cDNA and genomic sequences in *Oenothera* downstream of the stop codon are still highly conserved with the polypeptide sequences of other species and identical with the *Petunia* encoded polypeptide. The stop codon presumably precludes continuous translation of this sequence and therefore suggests the *Oenothera* mitochondrial genome to encode only rps19 pseudogenes. An intact (i.e. full length) RPS19 polypeptide required in the mitochondrial ribosome must thus be encoded by the nuclear genome and be imported from the cytoplasm.

Sequence analysis of individual cDNA clones from this rps19 pseudogene coding region revealed seven different nucleotide sequence divergencies between genomic and cDNAs (Figures 3 and 4). All were C to U alterations in the cDNA sequences and are thus attributed to RNA editing. Five of these editing events change the encoded amino acid, while two occur in third codon positions and remain silent. Two of the non-silent editings alter amino acid triplets downstream of the stop codon. The three non-silent editings upstream of the stop codon change amino acid codons highly conserved before editing to codon specificities not found in any of the other rps19 sequences of the species compared (Figure 3D). Two of the four amino acids altered by RNA editing in *Petunia* (15) are 'pre-edited' in the *Oenothera* genomic sequence. The other two residues changed by editing in *Petunia* are, although identically encoded in the *Oenothera* and *Petunia* genomes, not edited in *Oenothera* in any of the cDNA clones analysed. The most frequent editing event in the *Oenothera* cDNAs, however, is found at the same position as editing event four in the *Petunia* sequence (15). This silent editing alters a

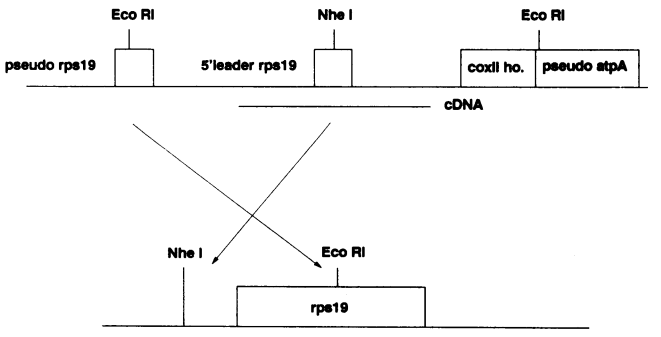

Figure 1. A genomic region in the mitochondrial genome of *Oenothera* contains numerous pseudogenes (top line). Overlapping with a truncated atpA gene lacking only the first nucleotide is a sequence with homology to coxII (13). Two sequence fragments are located upstream that are also found in another genomic region encoding a sequence with significant similarity to rps19 genes of other species (bottom line). At least part of the pseudogene cluster is transcribed as shown by cDNA clones.

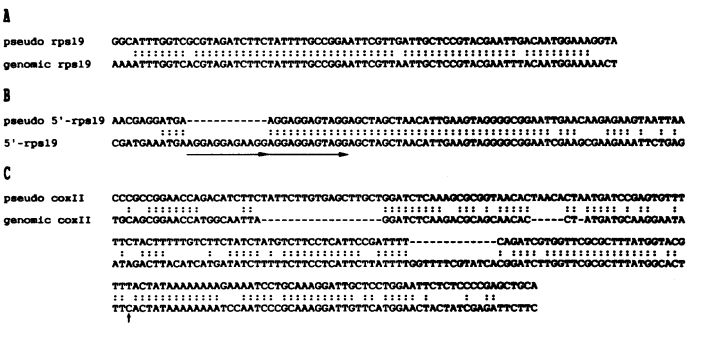

Figure 2. Alignment of the three sequence fragments with homology to coxII, 5'-rps19 and rps19 genes with the 'original' loci shows the different degrees of conservation. Identical nucleotides are indicated, dashes denote gaps introduced for improved alignment and the two horizontal arrows show a sequence duplication in the rps19 leader sequence. The vertical arrow indicates a nucleotide edited in the coxII mRNA and encoded in its edited version in the pseudogene (27). The atpA pseudogene sequence is identical with the atpA sequence downstream of the initiation codon (not shown).

serine codon from TCC to TCT. The adjacent C in this codon is also altered in some of the *Petunia* cDNA clones (editing site three; 15), but never without the silent exchange. The conserved silent editing site is altered with similar efficiencies in *Oenothera* and *Petunia*, with 10 of 14 and 6 of 8 cDNA clones edited, respectively.

Individual cDNA clones covering the rps19 pseudogene sequence in *Oenothera* mitochondria show highly divergent patterns of RNA editing and none of the cDNA clones is fully edited (Figure 4A). The most highly edited cDNA clone (no. 6 in figure 4A) showed 5 of the seven editing sites altered, the least edited clone (no. 12; Figure 4A, B) did not show any difference to the genomic sequence. The other intermediately edited clones display almost all possible permutations of edited and unedited sites suggesting the editing process to proceed undirectional.

RNA editing creates a stop codon in the rps3 coding sequence

Many of the rps19 pseudogene cDNA clones extend into the downstream rps3 coding sequence, nine clones contain the entire first exon of rps3 (Figure 4A). These cDNA clones probably represent the bona fide mRNAs of rps3, since even competitive cis- and trans-splicing between transcripts from the truncated and the complete loci would result in identical open reading frames

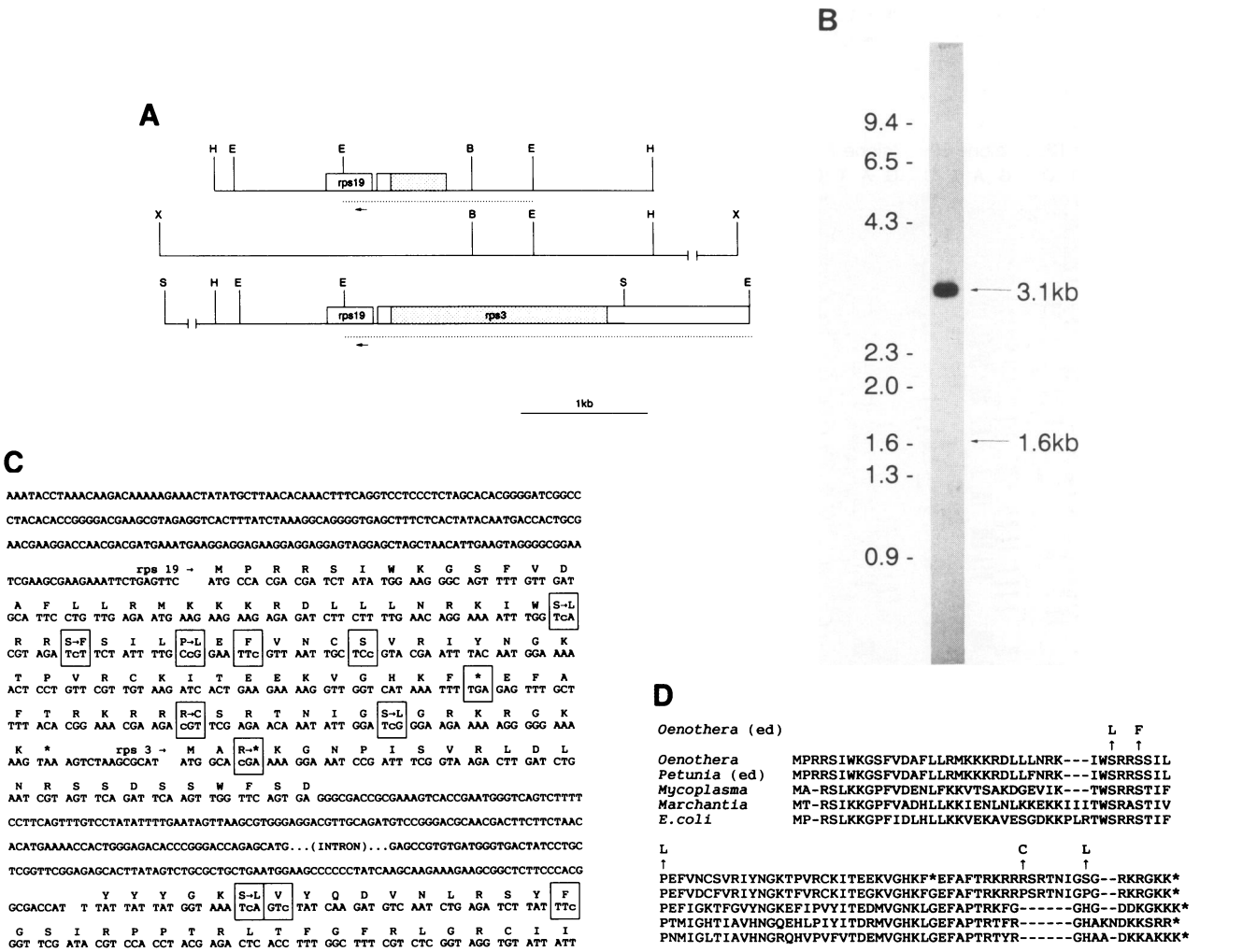

Figure 3. The sequence region with homology through the entire length of the rps19 coding region in *Oenothera* mitochondria contains a translational stop codon in genomic and cDNA sequences. Panel A: Two copies of this rps19 pseudogene are present in the mitochondrial genome due to a genomic rearrangement in the first intron (shaded) of the downstream located rps3 gene. The arrows show the position of the oligonucleotide (5′-GTGTAAAAGCAAACTCTCCAAATTTATG-ACC-3′) used for probing the Southern in panel B. The dotted lines indicate the two EcoRI fragments identified in the mitochondrial DNA in panel B. Some of the restriction recognition sites are indicated for BamHI (B), EcoRI (F), HindIII (H), SalI (S) and XhoI (X). Panel B: Hybridisation with an oligonucleotide specific for the complete rps19 shows the two copies of this sequence depicted in panel A to be the only arrangements detectable in the mitochondrial genome of *Oenothera*. The genomic fragment with the disrupted rps3 gene is present only in substoichiometric amounts, possibly encoded on one of the numerous small circular molecules present in the *Oenothera* mitochondrial genome. Panel C: Genomic and cDNA sequences of the rps19 and rps3 homologies show both sequences to be cotranscribed and edited. RNA editing sites observed in at least one cDNA clone are boxed with the edited nucleotide given in lower case and the resulting amino acid alteration indicated. Also boxed is the termination codon found in the rps19 homologous sequence in both genomic and cDNA clones. The sequences shown of the rps3 intron include the complete domains V and VI, diagnostic of group II introns. Panel D: Amino acid sequence alignment of the *Oenothera* rps19 full-length pseudogene with the rps19 protein sequences of other species (15, 19, 20, 28). For *Petunia* only the amino acids encoded in fully edited mRNAs are given (ed) with the residues divergently encoded by the genomic sequence shaded (15). The editings in the *Oenothera* sequence (arrows) occur in different positions and change several evolutionary highly conserved amino acid positions.

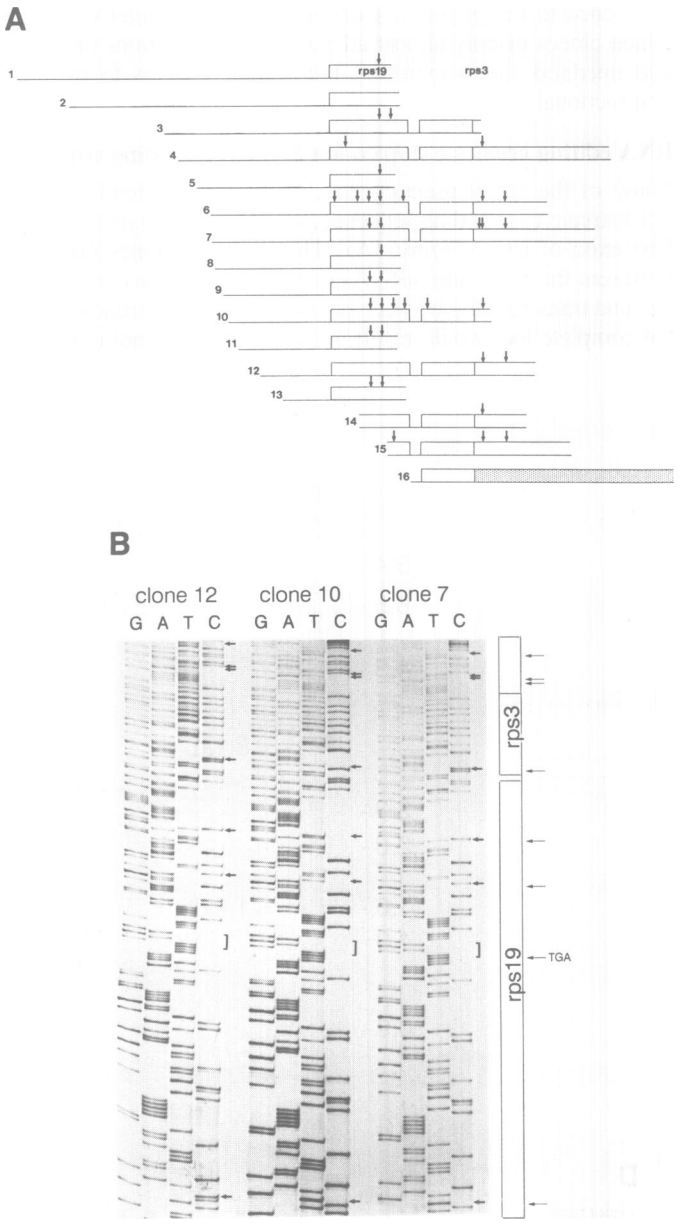

Figure 4. Individual cDNA clones show unique RNA editing patterns in the *Oenothera* rps19 sequence, but all contain the internal TGA termination codon. Panel A: Distribution pattern of the different RNA editing sites in the rps19 pseudogene region in individual cDNA clones (no. 1 through no. 16) shows unique editing frequencies for each site. There appears to be no order in the editing events and no direction of editing. The editing event in the first exon of rps3 creates a translational stop codon (Figure 3C). One of the cDNA clones is unspliced and contains the first intron of the rps3 gene (shaded). Panel B: Three cDNA clones demonstrate these different RNA editing patterns. The rps19 internal TGA termination codon is indicated and RNA editing sites are marked by arrows in the sequence autoradiographs and in the schematic arrangement of the genes in the right margin. The first editing site in rps3 is edited in clone no. 10, resulting in a TGA termination codon. Clone numbers refer to the clone identification in panel A.

for rps3. Excision of the first rps3 intron appears to proceed rapidly as suggested by the ratio of processed to unprocessed cDNA clones (8 to 1). Most of the other introns in *Oenothera* mitochondrial genes, like those in the nad1 and nad5 genes, show comparatively slower modes of processing with more unspliced than spliced cDNA clones found for these intron locations.

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                                    ↓
Oenothera     MARKGNPISVRLDLNRSSDSSWFSDYYYGKLVYQDVNLRSYFGSIRPPTR
Petunia       MARKGNPISVRLDLNRSSDSSWFS
Zea mays      MARKGNPISVRLDLNRSSDPSWFSDYYYGKLLYQDVNLRSYFSSIRPPTR
Marchantia    MGQKINPLGFRLGITQNHRSYWFANKKYSKVFEEDKKIRDCIELYVQKHI
E.coli        MGQKVHPNGIRLGIVKPWNSTWFAN---TKEF-AD-NLDSDFKVRQYLTK
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Figure 5. Alignment of the *Oenothera* rps3 amino acid sequence deduced from the cDNA open reading frames with the respective polypeptides of other species (14, 15, 19, 20). The *Petunia* sequence is shown as far as deducible from the available nucleotide sequence (15). Alignment of the maize sequence has been reassigned and the splice site identical in *Oenothera* and maize (14) is indicated by the arrow.

Sequence analysis of the rps3 genomic and spliced cDNA clones allows the precise determination of the splice site between exons a and b of the rps3 gene (Figures 3C and 4B). This splice site is located at the same position in the *Oenothera* and maize rps3 sequences (Figure 5; 14).

The maize mitochondrial editing site in the first exon of rps3 altering a CGG arginine codon to a TGG tryptophan codon three triplets before the splice site (14) is not required in *Oenothera* or *Petunia* mitochondria, where TGG codons are already genomically specified (Figures 3C and 5). The sixth amino acid codon of the second exon, edited also in maize (14), is found altered in all cDNA clones of *Oenothera*. A silent editing event 11 triplets downstream is likewise observed in all cDNA clones. Two further RNA editing events are uniquely observed in individual *Oenothera* cDNA clones, however. One difference is a silent editing event of the next C residue downstream of the always edited nucleotide in codon six of the second exon. The other sequence difference in cDNA clone no. 10 (Figure 4B) alters the third codon of the open reading frame from a CGA arginine triplet to a TGA termination codon.

Amino acid comparison of the three plant rps3 (partial) polypeptides deduced from the nucleotide sequences shows the genomic CGA triplet to be conserved in higher plant mitochondria (Figure 5). Extensive cDNA analyses in other plant species will be required to allow estimates of the distribution and relevance of this editing event. The amino acid sequence alignment proposed here (Figure 5) gives an improved similarity in the amino terminal region with the *E. coli* and the *Marchantia* chloroplast proteins (14, 19, 20). However, further analysis will be required to determine the rps3 polypeptide structure in plant mitochondria especially with the plant mitochondrial polypeptide being so much larger than the bacterial protein (14).

Translation stop codon by RNA editing in coxI mRNAs

A termination codon is found to be introduced by RNA editing in the 5'-terminal region of the cytochrome oxidase subunit I (coxI; 21) open reading frame in *Oenothera* mitochondria (Figure 6A). Analysis of RNA editing in the 5'-terminal region of the coxI open reading frame in 21 independent cDNA clones revealed six different types of RNA editing patterns, shown in figure 6B. Four types are represented by several of the independently derived cDNA clones, while two types are exemplified by single clones. Only one editing site was consistently found altered in all cDNA clones. This event is a non-silent change altering a CCG proline codon to a CTG leucine triplet. The two cytidines proximal on either side of this nucleotide are edited without any effect on the decoded amino acid sequence in two types of cDNA clones. Another silent editing is observed in one clone eleven nucleotides further downstream. The second C downstream from the site always edited is altered in another of the cDNA clones (type no.

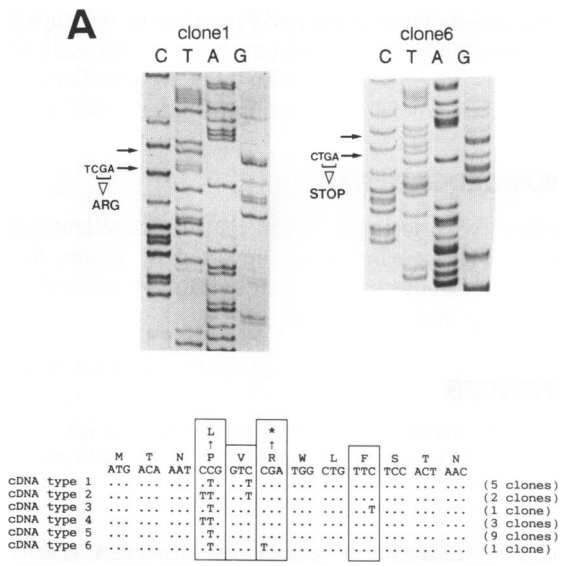

Figure 6. RNA editing in coxI transcripts of *Oenothera* mitochondria can lead to a TGA translational termination codon in the open reading frame. Panel A shows two cDNA sequences with one of two genomically adjacent C residues edited in each cDNA clone. Editing of the first nucleotide results in a third position silent exchange and leaves the CGA arginine codon unaltered in clone no. 1. Editing of the second C alters the CGA codon to a TGA stop codon in clone no. 6. The non-silent editing site upstream is edited in all cDNA clones (indicated by an arrow). Clone numbers refer to the cDNA types shown in part B. Panel B gives an overview of the classes of cDNA clones found with different editing patterns. The sites of differential editing in the 5'-region of the coxI transcripts are all in the vicinity of an editing site found altered in all cDNA clones.

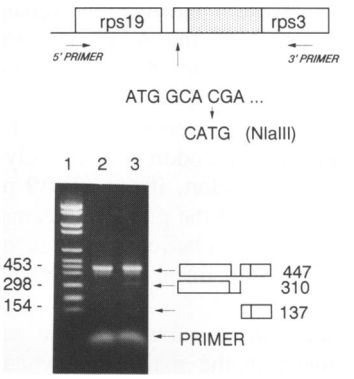

Figure 7. The percentage of molecules restrictable with NlaIII in the total PCR amplified cDNA population reflects the frequency of the disruptive rps3 termination codon (Figure 4B) introduced by RNA editing. This editing event creates a unique 5'-CATG-3' restriction recognition site for NlaIII. PCR products were amplified after reverse transcription of random primed total mitochondrial RNA between the 5'-primer (5'-ATACATATGCCACGACGATCTATATGG-3') and the 3'-primer (5'-GGTGGACGTATCGAACC-3'). The PCR products were split and electrophoresed untreated (lane 2) and after exhaustive digestion with NlaIII (lane 3). The schematic drawings indicate the uncut 447 bp amplification product spliced between rps3 exons 1 and 2 and the 310 bp and 137 bp fragments resulting from the NlaIII cut. Sizes of selected pBR 328/BglI and pBR 328/HinfI marker fragments (lane 1) are given on the left.

6 in figures 6A and 6B) and changes a CGA arginine codon to a TGA translational stop. This stop codon is located only five triplets from the initiation codon and thus will only allow a peptide of five residues to be synthesized if this mRNA is accessible for the ribosome at all.

DISCUSSION

The observation of strange RNA editings in a transcribed rps19 pseudogene and in coxI and rps3 mRNAs in mitochondria of the higher plant *Oenothera* reported here, allows several deductions on the RNA editing process in these organelles to be made. The independent introduction of stop codons in mRNAs of two different genes only few triplets downstream of the respective initiation codon is most likely the result of 'senseless' editing, examples of mistakes by the editing activity. Nevertheless these editings might also have some function in gene regulation in mitochondria.

If these editings were involved in mitochondrial gene expression, they could e.g. preclude readthrough by the ribosome leading to premature termination of translation. Another possible regulatory interference could be inhibition of ribosome binding, if these first few codons, three in the case of rps3 and six for coxI, participate in the initiation step of translation. Such editing could furthermore mark and destine these mRNA molecules for degradation, thereby regulating the pool of translatable mRNAs.

Other editing events, however, give much clearer indications of their potential role in regulation, like the creation of a translation initiation codon in nad1 mRNAs (5, 22) or an improved potential ribosome binding site in rps14 mRNAs (4). Editing in the nad1 intron sequences in *Oenothera* mitochondria appears to be required for intron excision and may thus be involved in regulating the levels of translatable mRNA molecules (5).

The unusual termination codons in the rps3 and coxI mRNAs are found only in a minor fraction of the mRNA population and thus presumably will affect synthesis of the encoded gene product only to a minor degree. However, this situation might be altered in other developmental stages of the plant. Several silent editings in the coxI region analysed here and in other mRNAs are like the termination codons detected only rarely in the cDNA clone population. Silent editings, however, do not impair translatability or the information content of the mRNA. They may be interpreted rather as tolerated editing errors in view of the — for translation — fatal errors of introduced termination codons, resulting in non-translatable mRNAs.

A crucial problem with the per definitionem rare editing 'mistakes' is the exclusion of cloning artefacts, when these events are observed in only a few individual clones. To obtain some estimate of the frequency of these events in the total mRNA population we analysed the percentage of molecules containing the aberrant termination codon in first exon of rps3 (Figure 7). RNA editing at this position generates a new restriction site for NlaIII, which is thus indicative for edited sequences. A small portion of the PCR products amplified from the total mitochondrial mRNA population can indeed be restricted with the enzyme, showing a subpopulation of the rps3 mRNAs to contain this stop codon. The result confirms the independent cDNA clone to correctly reflect a low abundance mRNA molecule containing this aberrant editing event.

This hypothesis of a detectable background of RNA editing mistakes in plant mitochondria is further supported by the RNA editing sites observed in the full-length rps19 pseudogene transcripts. The TGA termination codon found in genomic and all cDNA sequences makes translation of this sequence into an intact RPS19 polypeptide unlikely. In plant mitochondria TGA

codons function as efficient termination signals, since direct protein sequence analysis of the ATP9 polypeptide in wheat mitochondria showed the mature protein population to terminate at a TGA codon established by RNA editing (23, 9). This suggests the functional rps19 gene to be encoded in the nuclear genome, unless the TGA termination codon is selectively suppressed or used as a selenocysteine codon, if the RPS19 protein is at all necessary for the function of the plant mitochondrial ribosome. Detailed protein analyses will be required to determine whether the amino terminal RPS19 fragment encoded by the mitochondrial genome is synthesized at all.

An analogous situation was found for the gene coding for rps12, which is intact in the maize and wheat, but not the *Oenothera* mitochondrial genomes (24, 10). The remaining partial rps12 sequence in *Oenothera* is not edited at the single editing site present in this region in wheat. The rps19 sequence in *Oenothera*, however, is edited at seven sites differing in their patterns in individual cDNA clones. None of these editing events makes any sense in terms of resurrecting a genomically divergent codon to specify an amino acid conserved in evolution and presumably function. The rather opposite effect of RNA editing in this rps19 pseudogene by changing genomic encoded highly conserved triplets in the mRNA to specify evolutionary divergent amino acids suggests these editings to be senseless.

The different types of RNA editing mistakes found in this investigation occur in the vicinity of other editing sites or in the case of the rps19 pseudogene near 'former' editing sites. Such can be deduced from the comparison with the *Petunia* sequence, in which different, in evolutionary terms 'good' editing sites have been found (15). The alteration of editing sites in the pseudogene in *Oenothera* cannot be due to a loosened specificity of the editing process, but rather has to be attributed to a shift of the specificity from one site to other(s), since only one of the *Petunia* editing sites is also detected in *Oenothera*. Species-specific editing sites like in the rps13 genes in *Oenothera* and carrot may have been derived by an analogous new placement of the specificity determinant(s) (25). Although 'senseless' positions are now edited in the *Oenothera* rps19 pseudogene the editing activity has not lost its high discriminatory specificity, since the number of editing sites is still strictly limited to a few positions. This argument is consistent with the rare editing events in the 5' region of the coxI open reading frame, where only specific cytidines can be altered in the vicinity of the single 'good' editing at nucleotide 11 (Figure 6). However, distribution of the rare events in this sequence may alternatively be explained by a specificity gradient around this important, indispensable editing site (Figure 6B) that still allows some mRNA molecules to be edited at the nearby nucleotides with the editing efficiency diminishing with distance from this site.

From this analysis of faulty or 'bad' RNA editing events in *Oenothera* mitochondria we thus conclude that the specificity of the editing activity can be shifted from one nucleotide position to other(s) without losing its specificity. This possibility precludes primary or secondary sequence determinants in the vicinity of an mRNA to be solely responsible for qualifying a site to be edited as in the apolipoprotein B mRNA editing in mammalia (26). The specificity of RNA editing in plant mitochondria must reside in a determinant that can comparatively easily be redirected to other sequences.

A random switching of the RNA specificity to other nucleotide positions harmful for translation or processing, however, must be tolerated or prevented by the mitochondrion. Release of functional constraints as in the rps19 pseudogene will rapidly lead to senseless editings. Continuous evolutionary pressure appears therefore to be required to maintain the RNA specificity focused on editing sites crucial for function of the encoded product.

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