RNA Editing Site Recognition in Higher Plant Mitochondria

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RNA editing is a process by which genomically encoded cytidines are converted to uridines in plant mitochondrial transcripts. This conversion usually changes the amino acid specified by a codon and converts an "aberrant" residue to the evolutionarily conserved amino acid. The selection of the edited cytidine is highly specific. The *cis*-acting sequences for editing site recognition have been examined in ribosomal protein S12 (*rps12*) transcripts and in transcripts for a second copy of an internal portion of the ribosomal protein S12 (*rps12b*). *rps12b* was created by recombination at 7 and 9 nucleotide sequences that included editing sites I and IV of *rps12,* thus affording an opportunity to study the editing of chimeric transcripts with rearrangement very near C to U editing sites. Rearrangements downstream of editing site IV did not affect the editing of that sequence, while rearrangement upstream of editing site I ablated editing at that cytidine residue. Secondary structure predictions indicated that RNA structure did not correlate with the editing of these substrates. These results taken together with other studies in the literature suggest that RNA editing site recognition is primarily dependent on the 5′ flanking RNA sequence.

RNA Editing Modifies Genetic Information

A seemingly never-ending series of modifications of the central dogma of molecular biology has occurred over the last 40 years: RNA genomes, reverse transcription, RNA splicing, alternative splicing, self-splicing, *trans*-splicing, protein splicing, ribosomal frame shifting, and RNA editing. Among the most recent of these discoveries is RNA editing, which is defined as any change in the nucleotide sequence of an RNA that causes it to differ in sequence from the DNA that encoded it. RNA editing typically results in nucleotide changes that cause amino acid substitutions in the gene products or, alternatively, in nucleotide insertions which cause reading frame shifts. Thus RNA editing may direct changes in the amino acid sequence of a polypeptide and be responsible for the production of multiple gene products from a single "gene."

Since the original report of RNA editing in the kinetoplasts (mitochondria) of trypanosomes by Benne et al. (1986), this process has been described in a number of biological systems. The many types of RNA sequence changes that occur in RNA editing can be divided into two fundamental types of RNA modifications: nucleotide insertion/deletion editing and nucleotide conversion editing. Examples of nucleotide insertion/deletion editing include U insertion or deletion in kinetoplasts of trypanosomes (Benne et al. 1986; Feagin et al. 1988; Shaw et al. 1988); nucleotide and dinucleotide insertions, as well as C to U conversion, in the mitochondria of *Physarum* (Mahendran et al. 1994; Visomirski-Robic and Gott 1995); and G insertion in mammalian nuclear transcripts directed by the paramyxoviral RNA genome (Jacques et al. 1994).

Nucleotide conversion editing has now been described for transcripts from several systems: C to U (rarely U to C) in higher plant mitochondria (Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989); C to U in higher plant chloroplasts (Maier et al. 1995) and both C to U and U to C in bryophyte chloroplasts (Yoshinaga et al. 1996); C to U in mammalian nuclear transcripts for apolipoprotein B (*apoB*) (Chen et al. 1987; Powell et al. 1987); several nucleotide substitutions in tRNAs in *Acanthamoeba castellanii* mitochondria (Lonergan and Gray 1993); and A to I conversion in mammals, birds, amphibians, and flies (Bass and Weintraub 1988; Dabiri et al. 1996; Higuchi et al. 1993; Petschek et

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al. 1996; Polson et al. 1996). Thus many different processes cause nucleotide changes in RNAs and the many types of RNA editing result from mechanistically different types of RNA modification.

RNA Editing in Higher Plant Mitochondria and Plastids

RNA editing in plant mitochondria is ubiquitous among all the gymnosperms and angiosperms that have been examined (Gray and Covello 1993). Editing has recently been reported in bryophyte mitochondria and chloroplasts (Malek et al. 1996; Yoshinaga et al. 1996), placing the origin of the process earlier than previously thought. C to U editing has not been reported in bacteria or in organelles of algae or yeast and, therefore, C to U editing in plant organelles is apparently a derived characteristic.

In higher plant plastids, mRNA editing exclusively involves C to U substitutions. In higher plant mitochondria, C to U substitution is also the predominant form of editing, but rare cases of U to C modifications are also known. The total number of editing sites in the plastid RNAs is 25 to 30 (Maier et al. 1995), while in mitochondria it is estimated to be more than 1000.

In flowering plants, mitochondrial transcripts for almost every protein coding gene are edited, but the magnitude varies widely among transcripts for different genes. For example, only four nucleotides are edited in the 1533-nucleotide coding sequence of subunit 1 of ATP synthase (*atp1*) in *Oenothera* mitochondria, resulting in a 0.4% change of the deduced amino acid sequence (Schuster et al. 1991). At the opposite extreme, 21 nucleotides are edited in the 357-nucleotide coding sequence of subunit 3 of NADH-ubiquinone dehydrogenase (*nad3*) in maize mitochondria, resulting in a 15% change in the deduced amino acid sequence (Grosskopf and Mulligan 1996).

Most editing results in radical changes in the amino acid specified by a codon. The edited transcripts encode the evolutionarily conserved amino acid sequence of a polypeptide (Gualberto et al. 1989; Phreaner et al. 1996; Williams et al. 1998b), therefore editing is critical to the expression of the functional polypeptide (Bock et al. 1994; Phreaner et al. 1996). Although RNA editing may occur in any position of the codon, "silent" edits that do not affect the amino acid specified by the codon occur at a substantially reduced frequency (Gray and Covello 1993). Thus a primary consequence of C to U editing appears to be a genetic correction necessary for the production of the evolutionarily conserved amino acid sequence of a polypeptide.

C to U Editing Mechanisms in Plant Mitochondria

In principle, C to U editing could result from three types of mechanisms: (1) hydrolysis of the C4 amide of the cytosine base to directly convert cytidine to uridine (hydrolytic deamination); (2) removal of the cytosine base and replacement with uracil (transglycosylation); and (3) removal and insertion of a nucleotide monophosphate (nucleotide deletion/insertion). In nucleotide metabolism, cytidine is converted into uridine by cytidine deaminase ($CMP + H_2O \rightarrow UMP + NH_3$) and by a related reaction with cytosine, a nucleoside, that is catalyzed by cytosine deaminase. Several lines of experimentation indicate that C to U editing in plant mitochondria is a deamination reaction. Plant mitochondrial transcripts which were labeled with α-^{32}P-CMP by run-on transcription were shown to retain the radiolabeled α-phosphate as UMP residues (Rajasekhar and Mulligan 1993); thus eliminating mechanism 3, nucleotide deletion and insertion, which would exchange the α-phosphate. In addition, similar experiments with RNA labeled with 5′-^{3}H-cytidine confirmed retention of the pyrimidine ring in UMP residues, thus eliminating mechanism 2, transglycosylation (Blanc et al. 1995; Yu and Schuster 1995). These results strongly suggest that the C to U editing reaction in plant mitochondria is a deamination reaction.

The conversion of individual editing sites within a transcript appears to be random. Mature transcripts that show a high frequency of unedited sites exhibit a random distribution of the unedited nucleotides. Moreover, analysis of unspliced pre-mRNAs has demonstrated that nascent transcription products are incompletely edited and display a random distribution of editing site conversions (Sutton et al. 1991; Yang and Mulligan 1991; see also Gualberto et al. 1991). These data suggest that incompletely edited pre-mRNAs reflect intermediates in the editing process and that editing follows no processivity in plant organelles, in contrast to editing in the Typanosomatids.

The Origin of Editing Deaminase: A Derivative of Cytidine Deaminase?

A three-step model for the origin of RNA editing in plant organelles has been proposed by Covello and Gray (1993): (1) an editing activity appeared; (2) the capability to perform C to U changes at the RNA level permitted T to C mutations to accumulate in the organelle genome; and (3) numerous T to C mutations became fixed. Thus RNA editing became an obligatory step in gene expression. The editing apparatus essentially created a job for itself and then could not be eliminated.

The appearance of an editing activity as the initial step could have resulted from the modification of a related preexisting enzymatic activity. The evolution of APOBEC, the deaminase that edits mammalian nuclear mRNAs for apolipoprotein B100 (*apoB100*), appears to be an example of an editing enzyme evolving from an enzyme involved in nucleotide metabolism. *apoB100* mRNAs are edited at a single codon ($CAG^{gln} \rightarrow UAG^{ter}$) by deamination of a specific C (Bostrom et al. 1990; Hodges et al. 1991). C to U conversion creates a stop codon and produces a transcript with a truncated coding sequence that encodes the ApoB48 polypeptide. The *apoB100* gene directs the synthesis of two proteins with distinct roles in the formation of low-density lipoproteins (ApoB100, cholesterol-carrying) or chlyomicrons (ApoB48, triglyceride carrying). The catalytic subunit of the *apoB* mRNA editing enzyme is a 27 kDa member of the cytosine nucleoside/nucleotide deaminase family of enzymes (Navaratnam et al. 1993, 1995). In the evolution of a mononucleotide-specific cytidine deaminase to a polynucleotide (RNA)-specific deaminase with an editing function, the binding specificity would be expected to change such that the enzyme might either acquire an RNA binding domain or co-opt an additional subunit with RNA binding specificity.

The structure of cytidine deaminase from *E. coli* has been solved by X-ray crystallography, and the cocrystallization of the protein with a transition state analog has provided a detailed understanding of the structure of the active site and the interaction with the substrates (Betts et al. 1994). Cytidine and editing deaminases are zinc dependent and are known to have three conserved cysteine (or histidine) residues (↓) that function to coordinate the zinc atom (Figure 1) and a glutamate residue (⇓) that functions in a proton

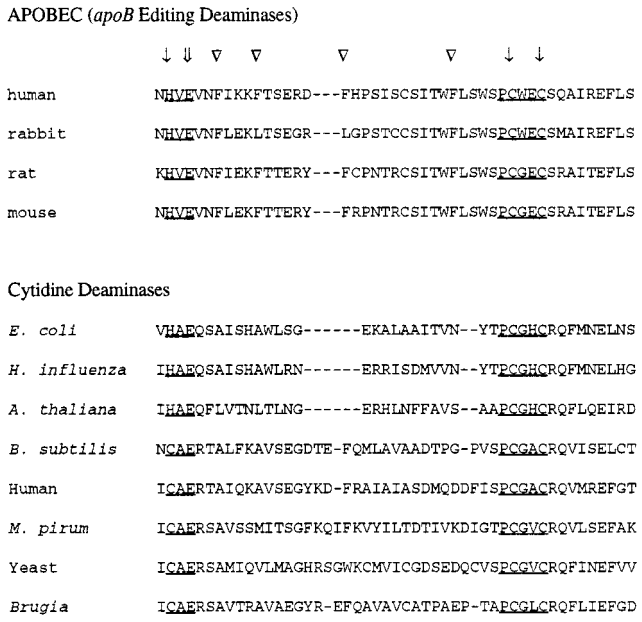

Figure 1. Sequence alignment of cytidine and C to U editing deaminases. Multiple sequence alignment was performed by ClustalW software (http://dot.imgen.bcm.tmc.edu,9331/multi-align/Options/clustalw.html). The single arrow (↓) indicates zinc binding residues. The double arrow (⇓) indicates the conserved glutamate residue involved in proton shuttling. The triangles (∇) indicate phenylalanine residues required by APOBEC for RNA binding. Species include bacteria (*Escherichia coli, Haemophilus influenzae, Bacillus subtilis*), mycoplasma (*Mycoplasma pirum*), yeast (*Saccharomyces cerevisiae*), nematode (*Brugia malayi*), and higher plant (*Arabidopsis thaliana*). The c/h a/v E and the P C g/w—C motifs are underlined. This figure is reprinted from Mulligan and Maliga (1998) with kind permission from the American Society of Plant Physiologists.

shuttle mechanism. A single zinc atom is coordinated by the cysteine (or histidine residue) of one α-helix (c/h a/v E motif, underlined) and the two cysteines of a second α-helix (P C g/w—C motif, underlined). The fourth ligand of the zinc atom is the substrate hydroxide molecule that attacks the C4′ carbon of the cytosine base. The conserved glutamate residue (⇓) facilitates the reaction through accepting and donating protons during the reaction (Betts et al. 1994).

The APOBEC deaminases have four phenylalanine residues (∇) that are not found in any of the other members of the cytidine deaminase family. Mutagenesis of these phenylalanine residues rendered APOBEC, the *apoB* editing deaminase, unable to bind RNA (Navaratnam et al. 1995). Additional mutations demonstrated that zinc and RNA binding are both required for the function of APOBEC, but can be separately ablated by specific mutations. Thus the evolution of APOBEC from the nucleoside/nucleotide family of deaminases probably occurred by acquisition of an RNA binding capacity from preexisting enzymes that were mononucleotide specific (Navaratnam et al. 1995).

RNA Modifications Specified by Antisense RNAs

Many RNA modification reactions utilize antisense RNA for site recognition, and there are many examples of RNA modification reactions that require either a *trans*-acting antisense RNA or *cis*-acting RNA sequences that participate in RNA duplex formation. 5′ splice site selection requires nine nucleotide complementarity of U1 snRNAs to the pre-mRNA (Zhuang and Weiner 1986), while 3′ splice site selection requires six nucleotide complementarity of U2 snRNA to the pre-mRNA (Zhuang and Weiner 1989). Hammerhead ribozymes can be directed in *trans* by 18-nucleotide complementarity (Cotten et al. 1989). Methylation of rRNAs is directed by the C/D box class of small nucleolar RNAs (snoRNAs) and utilizes 10- to 21-nucleotide complementarity (Kiss-Laszlo et al. 1998). Pseudouridylylation of rRNAs is directed by the H/ACA box class of snoRNAs and also utilizes antisense complementarity (Ni et al. 1997). U insertion editing utilizes antisense gRNAs in trypanosome editing reactions (Kable et al. 1996). Finally, double-stranded RNA adenosine deaminase utilizes double-stranded RNA for A to I deamination, which may be created by a secondary structure of a pre-mRNA (Higuchi et al. 1993).

The role of gRNAs in nucleotide insertion/deletion editing has been well characterized in the trypanosome system (Simpson and Thiemann 1995). Small antisense gRNAs direct U insertion or deletion in *trans* and include a 5′ anchor sequence with the antisense region, an "informational" sequence that corrects the region edited by that gRNA, and a 3′ poly-U tail (Figure 2). The 5′ anchoring sequence of the gRNA hybridizes with a downstream region of the preedited mRNA to form a duplexed RNA with 8 to 10 nucleotides of complementarity. Analysis by in vitro editing systems indicate that cycles of endonucleolytic cleavage, U deletion (Seiwert et al. 1996) or U insertion (Byrne et al. 1996; Connel et al. 1997; Kable et al. 1996), and RNA ligation occur in the reaction. gRNAs are the informational molecules in this system that direct U insertion and deletion dependent on the gRNA sequence.

A recent development in RNA modification is the elucidation of the role of snoRNAs in directing 2-O-methylation and pseudouridylylation of rRNAs (Balakin et al. 1996; Kiss-Laszlo et al. 1996; Ni et al. 1997). Hundreds of bases are modified in higher eukaryotic rRNAs by pseudouridylylation or by methylation. Specific nucleotides of these rRNAs are modified, but without an obvious consensus sequence or structural context to direct the modifi-

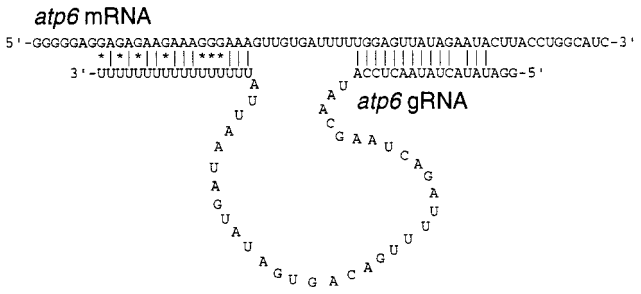

Figure 2. Structure of a Trypanosomatid mRNA/gRNA complex. The structure of the *atp6* mRNA/gRNA complex shows extensive base pairing in the 3′ region of the mRNA where the 5′ anchoring sequence of the gRNA has approximately 14 nucleotides of complementarity with the substrate RNA. In addition, the 3′ oligo-U tail of the gRNA is proposed to hybridize to the purine-rich preedited portion of the mRNA by Watson-Crick (|) and G-U (*) base pairing. This figure was modified from Figure 1 of Kable et al. (1996).

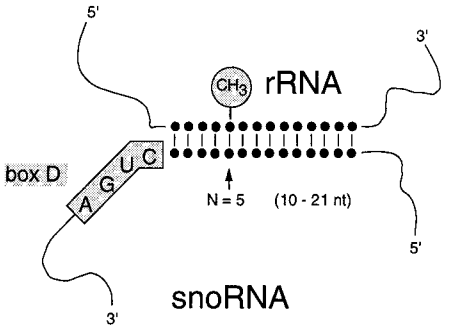

Figure 3. Structure of a rRNA methylation substrate. The C/D snoRNA includes a conserved D box which is adjacent to a region that is complementary to the rRNA methylation site. The methylation site of the rRNA is five nucleotides from the D box of the snoRNA. This figure was modified from Figure 1 of Bachellerie and Cavaille (1997).

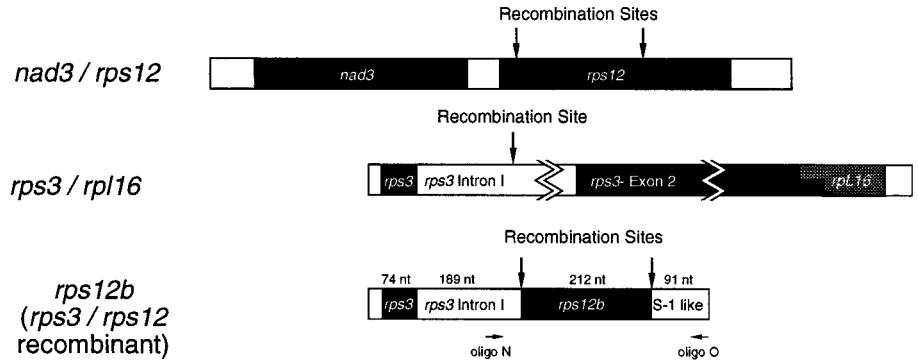

Figure 4. Recombination created two copies of *rps12* sequences in maize mitochondria. **(A)** The genomic organization of the *nad3/rps12* dicistronic transcription unit. **(B)** The genomic organization of the recombinant copy of *rps12,* referred to as *rps12b.* **(C)** The genomic organization of the *rps3/rpl16* dicistronic transcription unit. This figure is reprinted from Williams et al. (1998a) with kind permission from Kluwer Academic Publishers.

cations. Two distinct classes of snoRNAs have emerged that are 60–90 nt and include 10–21 nucleotides of complementarity to the rRNA and a pair of short conserved sequence motifs (3–7 nt). The C/D box snoRNAs direct 2-O-methylation and have conserved C and D boxes and a region of antisense complementarity to the region of the rRNA that flanks the methylated site (Figure 3) Mutagenesis of the rRNA and snoRNA demonstrated that the D box directs methylation exactly 5 nt upstream within the antisense region (Kiss-Laszlo et al. 1996). Thus the snoRNA selects the methylation site by antisense hybridization and a small conserved D box sequence of the snoRNA apparently serves as a landmark for the methylation apparatus to perform methylation 5 nt away. The H/ACA class of snoRNAs behaves in an analogous manner to direct pseudouridylylation of rRNA (Ni et al. 1997). These snoRNAs typically include two antisense sequence blocks of 5 to 6 nt that flank the pseudouridylylation site, as well as highly conserved ACA and H box sequences. Many snoRNAs are encoded within introns of a diverse array of nuclear-related and other genes and are apparently produced as by-products of RNA processing.

Identification of *cis*-Acting Elements in Mitochondrial Editing Sites

The mechanism and informational basis for the selection of individual nucleotides as editing sites is unknown. No consensus sequence at an editing site has been identified in either mitochondrial or chloroplast RNAs. Nucleotide sequence comparison of mitochondrial editing sites indicated that the edited cytosine usually does not have a purine, especially a guanine, as the 5′ nucleotide (Bonnard et al. 1992; Gray and Covello 1993). In addition, some editing sites bear similarities that could reflect different "classes" of editing sites (Gualberto et al. 1990, 1991). Apart from these simple trends, no specific sequences or secondary structural motifs have been identified from the hundreds of mitochondrial editing sites examined (Gray et al. 1992; Gualberto et al. 1990; Wissinger et al. 1992).

One plausible hypothesis for editing site specificity in plant organelles is that antisense RNAs with specificity information may be present, similar in principle to gRNAs or snoRNAs (Kable et al. 1996; Simpson and Theimann 1995). Searches for antisense RNAs using hybridization techniques have not been fruitful. Sense hybridization to RNA gel blots failed to detect antisense transcripts for *cox3* (Gualberto et al. 1990). In addition, "low stringency" RNase protection assays with sense *cox2* transcript failed to detect any novel protected antisense transcripts in various classes of size-fractionated mitochondrial RNA (Yang AJ and Mulligan RM, unpublished results). A tobacco plastid DNA sequence was identified with certain features possibly expected of a gRNA; however, mutagenesis of this sequence did not abolish editing of the expected site (Bock and Maliga 1995). Possible explanation for the failure of these approaches to detect gRNAs are that the extent of complementarity involved in the gRNA/RNA interaction may be limited; that the putative gRNAs are present in fleetingly small quantities; and that gRNAs might be of nuclear origin.

Several studies have reported that large RNA sequences were effectively edited when expressed in another transcript. A recombination created a chimeric fusion of *atp9* coding sequence with the *atp6* coding sequence in the C cytoplasm of cytoplasmic male sterile (*cms*) maize (Kumar and Levings 1993). The repeated sequence extended 19 nucleotides downstream of the edited C and at least 21 nucleotides upstream, and this editing site was effectively edited when expressed in either context. Another example was reported with wheat *cox2* sequences that were effectively edited when expressed as a 193-nucleotide sequence in *orf299* within the *nad3/rps12* transcription unit (Gualberto et al. 1991). In this case, all three of the *cox2* editing sites were converted; the upstream editing site had 49 nucleotides of identity 5′ to the editing site and the downstream editing site had 51 nucleotides of identity in the 3′ direction. In addition, the editing of chimeric transcripts in male fertile and male sterile lines indicated that some unusual differences in editing may exist. For example, in the chimeric *cms*-related coding sequence of *pcf* in Petunia, all of the *atp9* and *cox2* editing sites were effectively edited, except for one *cox2* editing site which had very minor RNA sequence differences in that region (Nivison et al. 1994).

In plant mitochondria, *rps12* and *rps3* transcripts and a recombinant transcript, *rps12b,* express *rps12* editing sites I and IV within the recombination sequences (Figure 4). Analysis of the editing status of these transcripts suggests that 5′ flanking sequences confer editing site recognition (Williams et al. 1998a). Editing sites I and IV of *rps12* are within small recombination sites (7–9 nt). Site I is only edited in the *rps12* context. The 5′ rearrangement at nt −7 of the *rps12b* context completely ablated editing (0 edited cDNAs/35 cDNAs sequenced). The *rps12b* context differed

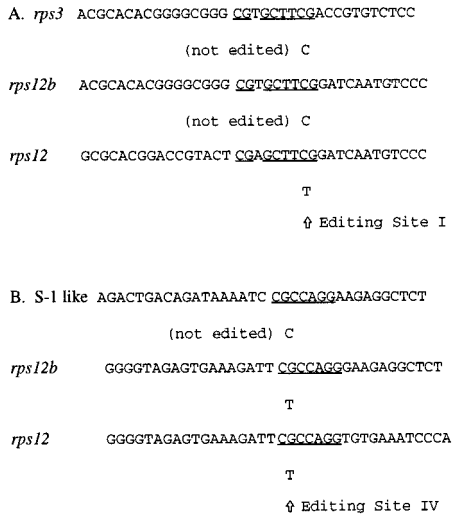

Figure 5. Comparison of recombinant DNA sequences and RNA editing sites. **(A)** Editing of transcripts with the 5′ recombination site of the *rps3* intron, *rps12b,* and *rps12* sequence. The DNA sequence of the nonanucleotide recombination site (underlined) is shown with the 5′ and 3′ flanking sequences of that context. The C to U conversion of *rps12* editing site 1 is indicated by an arrow (⇧) and a "T" underneath the edited nucleotide. Failure to detect C to U conversion is indicated by a "C" under the nucleotide. **(B)** Editing of transcripts with the 3′ recombination site of the 2.3 kb plasmid, *rps12b* and *rps12* sequences. The DNA sequence of the heptanucleotide recombination site (underlined) is shown with the 5′ and 3′ flanking sequences of that context. The C to U conversion of *rps12* editing site 4 is indicated by an arrow (⇧) and a "T" underneath the edited nucleotide. Failure to detect C to U conversion is indicated by a "C" under the nucleotide. This figure is reprinted from Williams et al. (1998a) with kind permission from Kluwer Academic Publishers.

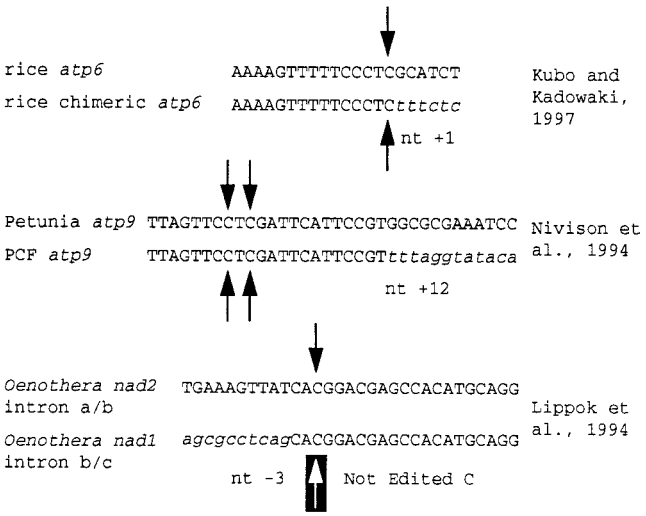

Figure 6. Editing of chimeric transcripts in plant mitochondria. The nucleotide sequence and editing status of repeated sequences in plant mitochondrial systems is shown. The region of nucleotide identity is shown in uppercase and regions of nonidentity are shown in lowercase italic text. Edited nucleotides are indicated by the solid black arrow pointing to the nucleotide, and the nucleotide that shows no editing is the white arrow in a black rectangle.

from the *rps12* editing site at the −5 nucleotide position (A to T change) and in the 5′ sequences upstream of the recombination site at nt −7 (Figure 5). The 3′ sequences were identical downstream of the editing site for more than 200 nucleotides (Figure 5). These data suggest that the sequence specifying an editing site must be larger than the nonanucleotide recombination sequence. In addition, it suggests that RNA sequence immediately around and extensively downstream are not sufficient for editing site recognition.

The 3′ recombination site is a heptanucleotide sequence containing editing site IV of *rps12* (Figure 5). This sequence is not edited when expressed as a transcript from the 2.3 kb plasmid. The 2.3 kb plasmid sequence differs from *rps12* immediately upstream and 6 nucleotides downstream of editing site IV (Figure 5). In contrast, the heptanucleotide sequence is edited when expressed in the *rps12b* context. The *rps12b* sequence is identical to *rps12* for 203 nucleotides upstream from the editing site, but diverges downstream of the 3′ recombination site (Figure 5). These data suggest that the RNA sequence upstream and immediately around the editing site is sufficient to direct editing site recognition. The editing of these recombinant *rps12b* transcripts suggest that 5′ sequences are critical to editing site recognition, while 3′ sequences may not be important.

The importance of 5′ flanking sequences in conferring editing site specificity in plant mitochondria is indicated in two additional studies (Figure 6). The editing of repeated sequences within intron a/b of *nad2* and intron b/c of *nad1* indicated that nucleotides −2 to +45 were not sufficient to confer editing site recognition (Lippok et al. 1994); however, a chimeric rice *atp6* editing site is converted when nucleotide +1 and downstream are completely divergent (Kubo and Kadowaki 1997). These reports, taken together with the results presented in this article, support a model of RNA editing site recognition based on 5′ flanking sequences that extend upstream of nucleotide −5, but 3′ flanking sequences are apparently relatively unimportant.

The *cis*-acting sequences in plastid C to U editing have been analyzed in vivo by introduction of modified editing substrates. Introduction of recombinant editing sites with deletions in the 5′ or 3′ flanking sequences have demonstrated that a 22-nucleotide sequence around the *psbL* site (nucleotide −16 to +5) is sufficient to direct efficient editing of the site (Chaudhuri and Maliga 1996). Similar results demonstrated the importance of adjacent 5′ flanking sequences of some of the *ndhB* editing sites; however, the editing of other *ndhB* editing sites behaved differently when 5′ or 3′ deletions were tested (Bock et al. 1996, 1997). Expression of chimeric plastid transcripts with the *psbL* editing site resulted in the specific decrease in the extent of editing of *psbL* transcripts and suggests that editing site specificity factors are limiting in *psbL* editing (Chaudhuri and Maliga 1996). The similarity of the results of deletion analysis of the *cis*-acting sequences in the plastid editing system and the analysis of recombinant mitochondrial sequences in this and other studies suggests that plastid and mitochondrial editing systems may have very similar mechanisms and molecules for the recognition of C to U editing sites.

Is Secondary Structure Involved in Editing Site Recognition?

RNA sequence modification could affect RNA editing substrates through interaction of the editing apparatus with either primary RNA sequence or with RNA secondary structure. In order to analyze the possible effects of RNA structure on editing substrates, regions around the recombination sequences were analyzed by prediction of RNA secondary structure. Structural predictions of RNA sequences were performed with the 72 and 101 nucleotides encompassing the editing sites (Figure 7).

The secondary structure predicted around editing site I of *rps12* (Figure 7A) and around the comparable sequence of *rps12b* (Figure 7B) indicated that 25 nucleotides of a stem-loop structure were identical between the two structures, including most of the stem and loop nucleotides.

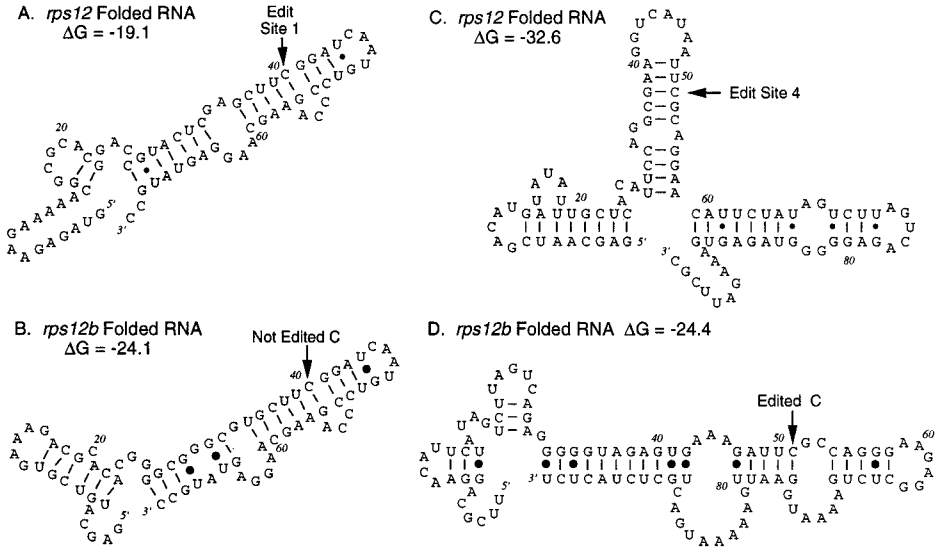

Figure 7. Predicted RNA structures of *rps12* and *rps12b* sequences. RNA secondary structure prediction was analyzed by mfold (version 2.3) by Zuker and Turner, courtesy of Dr. Michael Zuker's homepage (http://alfredo.wustl.edu/~zuker/rna/form1.cgi). RNA sequences were folded with temperature set at 30°C. Similar structures were obtained with either the 72 or 100 nucleotides flanking the recombination sites. **(A)** Predicted secondary structure of a 72-nucleotide RNA encompassing editing site 1 of *rps12.* The arrow indicates the edited nucleotide at editing site 1. **(B)** Predicted secondary structure of a 72-nucleotide RNA encompassing the 5′ recombination site of *rps12b.* The arrow indicates the nucleotide within the nonanucleotide repeat that is not edited in *rps12b.* **(C)** Predicted secondary structure of a 101-nucleotide RNA encompassing editing site 4 of *rps12.* The arrow indicates the edited nucleotide at site 4. **(D)** Predicted secondary structure of a 101-nucleotide RNA encompassing the 3′ recombination site of *rps12b.* The arrow indicates the nucleotide within the repeated sequence that is edited in *rps12b.* This figure is reprinted from Williams et al. (1998a) with kind permission from Kluwer Academic Publishers.

Although the overall structures predicted for these two RNAs were very similar, with a two-stem structure and similar locations of bulges in the major stem, the *rps12* RNA sequence was edited (Figure 7A), while the *rps12b* sequence was not edited (Figure 7B). Thus, although the secondary structure predictions indicate similar structures can be attained by these two transcripts, only the *rps12* transcript was an editing substrate.

The secondary structures predicted around editing site IV of *rps12* (Figure 7C) and around the comparable sequence of *rps12b* (Figure 7D) were very different. Figure 7C shows that editing site IV was predicted to be in the middle of a stem in a small stem loop, while Figure 7D shows that the edited C of the *rps12b* sequence was near a large bulge in a long stem. Despite the dissimilarity of the predicted structures around the edited nucleotides, the C in each RNA sequence was edited to a similar extent.

These observations suggested that substrate RNA structure may not be important in editing site recognition. First, the predicted *rps12* and *rps12b* RNA structures were similar for the sequences around the 5′ recombination site (Figure 7A,B); however, only the *rps12* RNA was edited. Second, the predicted RNA structures were different around the 3′ recombination site (Figure 7C,D); however, both RNAs were edited. Thus, in neither case did the secondary structure prediction correlate with the selection of these transcripts as substrates for C to U editing. However, small differences in the structures do exist (Figure 7A,B), and it is certainly possible that these may be critical for recognition of an editing substrate.

Conclusion

These studies indicate that editing site specificity in mitochondrial transcripts is directly influenced and determined by recombination and changes in RNA sequence. Chimeric transcript analyses have shown that editing is not affected by genetic recombination events as long as the primary sequence around the editing site is maintained. Furthermore, increasing evidence indicates that sequences immediately upstream of an editing site are crucial for site recognition. If trypanosome-like guide RNAs exist in plants and are required for editing site recognition, then these 5′ nucleotides may act as *cis*-acting elements. The results of several investigations into the editing of recombinant RNAs in plant mitochondria suggest that the region of editing site recognition is apparently no more than six nucleotides downstream but more than five nucleotides upstream of the editing site. The identification of *cis*-acting recognition sequences in RNA editing is an important aspect of editing in plant organelles and may lead to predictions about the mechanism and identification of macromolecules involved in RNA editing in plant organelles.

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