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Involvement of 5′ flanking sequence for specifying RNA editing sites in plant mitochondria

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Abstract Unsuccessful insertion of foreign DNA into plant mitochondrial genomes has hindered scientific evaluation of *cis*-elements needed for RNA editing. Both a normal *atp6* gene and a chimeric *atp6* sequence are present in rice mitochondria. The chimeric *atp6* contains one-half of the normal *atp6* sequence in its 5′ portion and an unknown sequence in its downstream portion. The C-nucleotide at position 511, located just upstream of the unknown sequence recombined in the chimeric *atp6* sequence, is edited, as are other possible editing sites upstream from position 511. We report here that the 5′ sequence adjacent to the editing site of *atp6* contains *cis*-information required for RNA editing and that the 3′ sequence flanking the editing site provides little contribution to editing-site recognition.

Key words: RNA editing; *atp6*; Mitochondria; Chimeric gene; *cis*-Element

1. Introduction

RNA editing, whereby DNA-encoded information is altered after transcription, was first discovered in trypanosomes [1]. RNA editing has since been found in viruses, fungi, mammals, and plants [2]. RNA editing of nucleotide insertion and/or deletion is known to occur in trypanosomes, a fungus (*Physarum polycephalum*), a group of viruses (paramyxovirus), and vertebrate mitochondria [2]. RNA editing by nucleotide substitution occurs in viruses (HIV and hepatitis virus), fungi (*Acanthamoeba castellani*, *P. polycephalum*, and *Spizellomyces puncatatus*), mammals, and plants [2,3]. RNA editing in fungi, mammals, and plants is a post-transcriptional process, whereas, G-nucleotide insertion in paramyxovirus is a co-transcriptional process [4]. In trypanosomes, guide RNA (gRNA) is involved in specifying the RNA editing site, and RNA editing occurs in a 3′-to-5′ orientation [5]. In mammalian intestines, a site-specific cytosine deaminase, for which the mooring sequence has been identified, is involved in editing of *apoB* mRNA [6]. A double-stranded RNA-specific deaminase is involved in editing of the β-subunit of glutamate receptors in mammalian brains [7] as well as antigenomic RNA in the hepatitis delta virus [8]; the secondary structure of RNA is important in these types of editing.

In plant mitochondria, RNA editing is widely observed [9–11] among lower and higher plant species [12], although the extent of RNA editing differs among species. In most cases, C-nucleotides are edited to U-nucleotide in RNA, with very few U-to-C transitions [13]. Cytosine deamination is involved in plant RNA editing [14–16]. Extensive cDNA analysis shows no strict polarity of RNA editing [17]. High specificity for RNA editing sites suggests involvement of individual *cis*-sequences in determining each editing site, because no apparent consensus sequence or secondary structure has been found around the sites.

For some editing sites, sequence similarities among mitochondria and chloroplasts have been identified [18]. This raises the possibility that mitochondria and chloroplasts share the same component(s) involved in RNA editing. Similarities between mitochondrial and chloroplast RNA editing include C-to-U transitions, lack of polarity, and post-transcriptional modification. A *cis*-element of 22 nucleotides that is required for chloroplast *psbL* editing was evaluated by a transplastomic approach [19]. This sequence includes 16 nucleotides upstream and 5 nucleotides downstream of the editing site. A similar condition has been reported for chloroplast *ndhB* editing, in which an essential element is located in the 12 nucleotides upstream region and 2 nucleotides downstream region of the editing site [20]. Recent works reveal that a chloroplast sequence that migrated to mitochondria is not edited in the mitochondria [21], nor is the mitochondrial sequence transformed into chloroplasts edited in the chloroplasts [22]. These results suggest involvement of organelle-specific factor(s). Hence, information within the *cis*-element that is necessary for chloroplast editing may not be directly applicable to *cis*-element information in mitochondrial editing. Insertion of foreign DNA into the mitochondrial genome would be an effective method to discern a sequence specifying the RNA editing site and to elucidate RNA-editing factors. However, no transformation system for plant mitochondria has been established to date, nor is in vitro RNA-editing analysis for *cis*-element evaluation available for plant mitochondria. Under these circumstances, analysis of chimeric genes should facilitate understanding of the RNA-editing process in plant mitochondrial genomes.

A chimeric sequence for ATPase subunit 6 (*atp6*) resulting from a single recombination event through a seven-nucleotide sequence (TTCCCTC) has been identified along with a normal *atp6* sequence in rice mitochondria [23]. Editing sites of *atp6* sequences in *Oenothera*, *Petunia*, maize, sorghum, and rapeseed are known [24–28]. Comparison of rice sequences with edited *atp6* sequences suggests that RNA editing occurs in both normal and chimeric *atp6* transcripts in rice (Fig. 1). We used this chimeric *atp6* sequence to assess potential *cis*-elements necessary for RNA editing. Interestingly, the recombined sequence is located just downstream of the C-nucleotide at position 511, where corresponding C-nucleotides in *Oenothera* and *Petunia* are edited and the same nucleotide position is occupied by T in maize, sorghum, and rapeseed. The 5′ sequence flanking position 511 originated from *atp6* but the 3′ flanking sequence is entirely replaced. Complete se-

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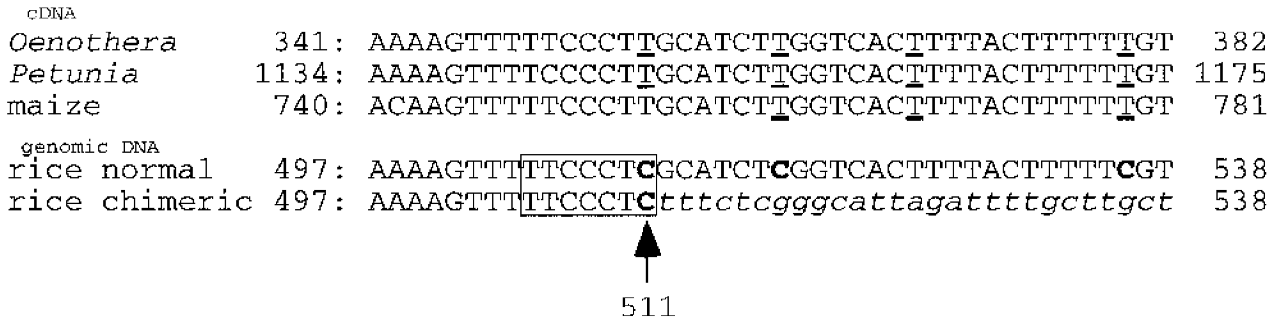

Fig. 1. Alignment of portions of *atp6* sequences in different plant species. RNA editing sites in *Oenothera*, *Petunia*, and maize cDNA sequences [23–26] are underlined. Potential editing sites of normal and chimeric *atp6* genomic sequences are presented in bold font. The arrow indicates position 511 in normal and chimeric *atp6* nucleotide sequences. The box encloses 7 nucleotides involved in a recombinational event [22]. A downstream sequence to position 511 is given in lower-case, italic font. Numbering corresponds to that used in previous reports [23–26].

quence conservation of chimeric *atp6* against its ancestral counterpart sequences suggests that recombination happened recently [23].

Because normal and chimeric *atp6* sequences are present and transcribed in the same mitochondria [23], RNA editing of these two sequences is likely to be carried out by the same editing components. This presents an opportunity to test involvement of the 3′ adjacent sequence in plant mitochondrial RNA editing. Hence, chimeric *atp6* allowed us to evaluate the importance of the 3′ flanking sequence in editing-site recognition.

2. Materials and methods

2.1. Isolation of mitochondrial RNA

Mitochondrial RNA was isolated from young seedlings of rice (*Oryza sativa* L., cms-Bo Taichung 65) as described previously [23]. Plant material (cms-Bo Taichung 65) has a cytoplasm derived from indica rice cultivar 'Chinsurah Boro II'.

2.2. cDNA synthesis, PCR amplification, and nucleotide sequence analysis

cDNAs were synthesized as previously described [29] and amplified by PCR according to a standard protocol. Absence of DNA contamination during RNA preparation was confirmed by control experiments in which water was used in place of reverse transcriptase. Amplified products were cloned into a pBluescript vector (Stratagene), sequenced, and analyzed as previously described [30].

2.3. Oligonucleotide primers

The sequences of the oligonucleotide primers were as follows:

P1, 5′-AAGATGGGGAATTCCAATGAATTT-3′
P2, 5′-GCTCCTCGTTTGAATTCAATTATG-3′
P3, 5′-TCATTCACAAATGAATTCTTG-3′
P4, 5′-GCAAATAAACGAATTCCTGAG-3′
P5, 5′-CTACTCTGAATTCAAGCTAGT-3′

These primers were designed based on the rice *atp6* sequence [23]. An *Eco*RI recognition site (underlined) was introduced into these oligonucleotides for convenient cloning.

3. Results and discussion

To determine whether position 511 is edited in normal *atp6* rice transcripts, cDNAs containing the entire coding region of *atp6* were amplified by PCR using primers P1 and P2, and cloned (Fig. 2). Nucleotide sequence determination of three independent clones, and subsequent comparison between cDNAs and genomic sequences, revealed 17 C-to-T modifications within the normal *atp6* open reading frame. Editing occurred at positions 13, 338, 365, 407, 511, 518, 536, 544, 551, 552, 745, 767, 809, 946, 953, 962, and 1000 (Fig. 2B). With the exception of position 13, these sites were located in an evolutionarily conserved region. Position 13 was located in the N-terminal extension. It has been suggested that this extended region is a precursor and is cleaved off post-translationally to generate a mature polypeptide [31]. All 17 editing sites are expected to result in amino-acid changes specified by codons, which mostly increase the amino-acid similarity of *atp6* to those of different plant species. The C-nucleotide at position 511 was edited.

To address occurrence and frequency of RNA editing in chimeric and normal *atp6* in detail, cDNA sequences that included position 511 were amplified with primers P3 and P4 for 470 bp of normal *atp6* transcripts, and with primers P3 and P5 for 404 bp of chimeric *atp6* transcripts. Ten and 69 clones were analyzed for normal and chimeric cDNAs, respectively. Two clones out of 10 were fully edited in normal *atp6* transcripts (Fig. 3A). Three clones were partially edited, and editing at 511 was observed in 40% (four out of ten) clones. Five clones were not edited at all.

Chimeric *atp6* transcripts were edited at four sites (positions 338, 365, 407, and 511) (Fig. 2B). These chimeric *atp6*-editing sites were identical to those of normal *atp6*. It is noteworthy that the C-nucleotide at position 511 in the chimeric transcript was edited, although the downstream sequence of the edited nucleotide was totally different from the original *atp6* sequence. Ten cDNA clones were fully edited, 19 clones were

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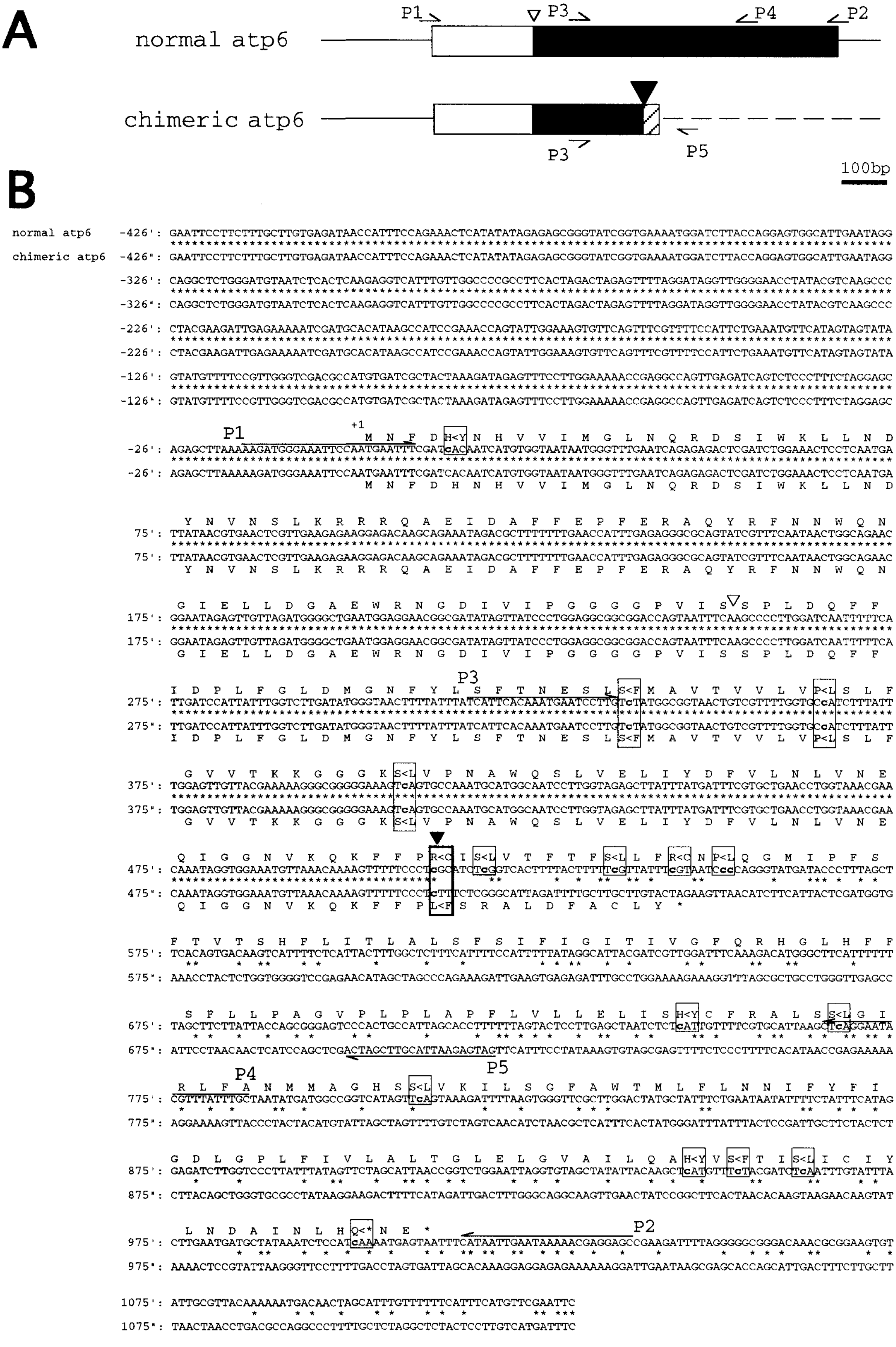
Fig. 2. RNA editing analysis of normal and chimeric *atp6* sequences from rice mitochondria. A proposed processing site [31] and recombinational event positions are shown by open and filled triangles, respectively. (A) Schematic representation of analyzed sequence. Open and filled boxes represent N-terminal extensions and evolutionarily conserved regions of *atp6*, respectively. A hatched box and a dotted line indicate an unknown sequence fused to the *atp6* core region. Boxes represent open reading frames. Relative positions of primers are indicated. (B) Nucleotide sequence alignment of normal (top) and chimeric (bottom) *atp6* sequences. Deduced amino acid sequences are shown. Asterisks indicate nucleotide sequence similarities between normal and chimeric atp6. C-Nucleotides subject to RNA editing are given in lower-case, bold font. Amino-acid transitions caused by editing are shown in boxes to the right of genomically encoded amino acids. A thick box indicates the codon containing editing site 511. Primer positions are indicated.


A
normal atp6
chimeric atp6
P1
P3
P4
P2
P3
P5
100bp
B
normal atp6
chimeric atp6
+1
P1
P3
P4
P5
P2

partially edited, and 40 clones were not edited (Fig. 3B). Fifteen out of 69 clones (22%) were edited at position 511. No editing event was observed within the sequence of unknown origin, suggesting that it was not derived from a protein-coding sequence. These results suggest that the 5′ sequence adjacent to the editing site is sufficient to specify editing site 511 and that the 3′ sequence provides little or no contribution.

The chimeric *atp6* sequence in maize, designated as C-*atp6*, is a triple fusion of a 39-bp fragment of the *atp9* sequence, an 806-bp fragment of *atp6*, and a sequence of unknown origin [26]. The *atp9* and *atp6* sequences of C-*atp6* are edited at sites corresponding to normal *atp9* and *atp6* mRNAs of other plant species. Similar results have been observed in transcripts of chimeric genes; wheat *orf299*, *Petunia pcf*, and rapeseed *orf224* [17,32,33]. In contrast, sugar beet *orf324* is not edited despite similarities to original sequences [34]. Kubo and Mikami inferred that three sites of nucleotide substitution in the vicinity of the editing site might prevent *orf324*-editing [34]. Taken together, these results suggest that editing may occur in a sequence-specific manner and that only a small fraction of duplicated sequences in the vicinity of an editing site could be *cis*-elements recognized by the *trans*-acting factor(s) that specify editing of transcripts of native genes.

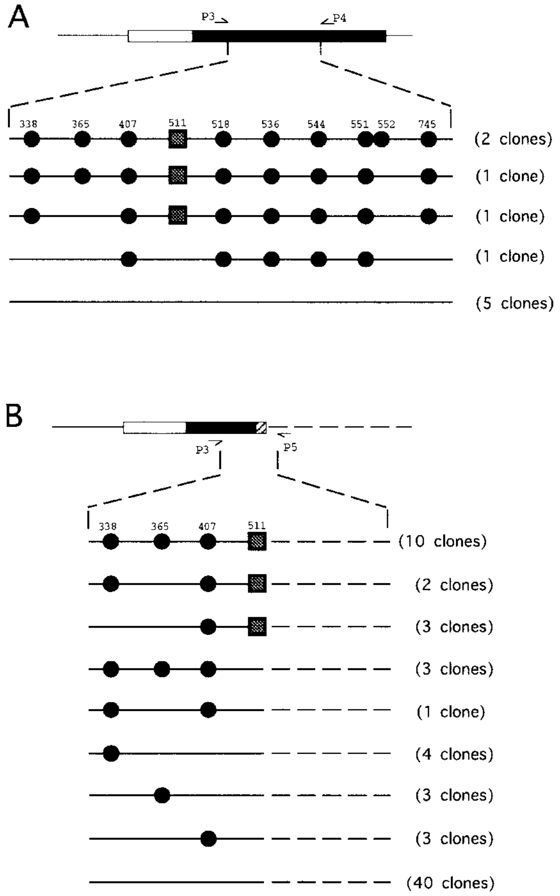


Fig. 3. Editing patterns of individual cDNAs from normal and chimeric *atp6* transcripts. Editing sites in cDNA portions of normal and chimeric *atp6* are schematically shown in panels A and B, respectively. Editing positions correspond to those in Fig. 2. Solid and dotted lines represent a normal *atp6* sequence and a sequence of unknown origin, respectively. Editing position 511 is represented by dotted squares, and other editing sites by filled circles. The numbers of analyzed clones are shown in parentheses.

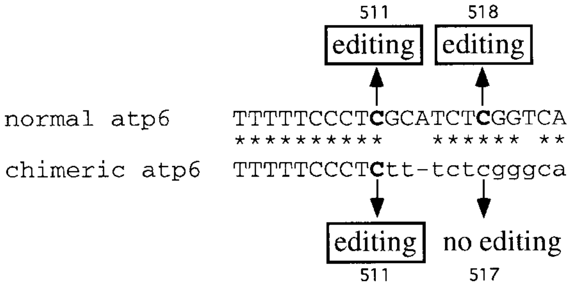


Fig. 4. Nucleotide sequence alignment of normal and chimeric *atp6* around the recombined region. Occurrences of RNA editing are shown. Asterisks indicate identical nucleotides. A dash is introduced to maximize nucleotide sequence correspondence.

Although chimeric *atp6* is apparently generated by a single recombination event [23], some extent of sequence similarity can be observed around position 517 between normal and chimeric *atp6* (Fig. 4). When the two sequences were compared, the 5′ sequence flanking position 517 of chimeric *atp6* was similar to that of normal *atp6* except for one nucleotide deletion and two nucleotide alterations (−4 to −6). The 3′ sequence flanking position 517 in chimeric *atp6* had one nucleotide difference out of five nucleotides, at position +3. However, cDNA analysis showed no RNA editing at position 517. Rapeseed *orf224* has an RNA editing site derived from a part of *rps3* that has a 37-bp 5′ flanking sequence and a 5-bp 3′ flanking sequence [33]. If the RNA-editing machinery in plant mitochondria is the same in all plants, then the 3′ flanking sequence required for editing site recognition extends as far as 5 bp [33]. The failure of RNA editing at position 517 may be caused by nucleotide differences around positions −4 and −6 and/or position +3. This lack of editing at position 517 also supports our speculation that the 5′ flanking sequence is greatly involved in specifying the RNA editing site in *atp6* transcripts.

In conclusion, analysis of mitochondrial chimeric *atp6* transcripts strongly suggests that the 5′ sequence upstream of editing sites is sufficient for recognition of such sites (Fig. 5). In trypanosomes, gRNAs specifically bind to the 3′ downstream sequence of the mRNA editing sites [4]. The mooring sequence of cytosine deaminase for *apoB* editing is located at the 3′ region of the editing site [6]. The double-stranded structure around the editing site is important for *gluB* editing [7]. Such observations demonstrate the involvement of the 3′ sequence to editing-site recognition. In contrast, it is unlikely that the 3′ downstream sequence is recognized as a *cis*-sequence in plant mitochondria (Fig. 5). Our results indicate that the 3′ flanking sequence is of little or no importance in defining mainly the *cis*-sequence during RNA editing in plant mitochondria. If a common editing mechanism is used to recognize sequences in all plant mitochondria, then the recognition site should not be far from the editing site because editing occurs in the *atp9* portion of C-*atp6* that has only 39 nucleotides in a 5′ flanking sequence [26]. The possibility that a sequence around the editing site may also form a secondary structure is unlikely, because computer analysis failed to detect identical secondary structures around editing sites of the *atp6* sequence (data not shown). Moreover, editing occurs

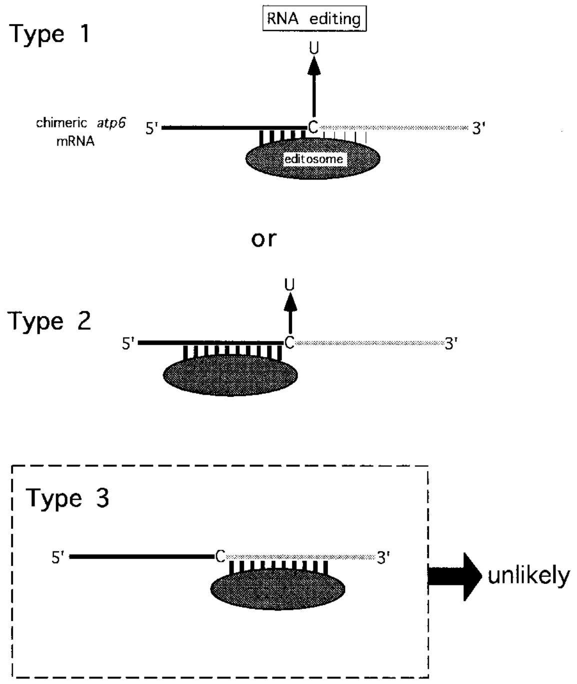


Fig. 5. Proposed models of *atp6* RNA editing. Horizontal thick and dotted lines represent an *atp6*-derived sequence and a sequence of unknown origin, respectively. The shaded ellipse represents a hypothetic editosome. Nucleotide interactions between *atp6* mRNA and the editosome are shown by vertical lines. Vertical thick and thin lines represent strong and weak recognition for the editing site, respectively. The type 3 model of RNA editing of plant mitochondria seems unlikely because chimeric *atp6* has a non-homologous sequence at the 3′ flanking region that is subject to RNA editing.

in a highly sequence-specific manner irrespective of secondary structure.

Because no evaluation method for the 3′ flanking region with different sequences in transformed mitochondria has been developed for plants, we can not completely exclude the possibility of involvement of the 3′ flanking sequence as a distal modulator in a loose sequence context. At present, use of chimeric gene sequences to analyze RNA editing in plant mitochondria may be the only way to understand *cis*-element involvement. In the future, development of an in vitro editing condition and/or transformation of foreign DNA into mitochondrial genomes would be valuable in minimizing the *cis*-element of plant mitochondrial RNA editing and elucidating *trans*-acting factor(s).

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