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# Different patterns in the recognition of editing sites in plant mitochondria

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## ABSTRACT

**Higher plant mitochondrial mRNAs are extensively modified by highly specific C-to-U conversions. However, the determinants of recognition specificity are, to date, unknown. Here, we analyse the *cis*-elements involved in the recognition of two editing sites in a *cox2* gene in wheat mitochondria. A minimal region of 23 nt was found to be involved in recognition of the editing site C77, similar to our previous report for site C259. These regions were correctly recognized by the mitochondrial editing machinery when placed elsewhere in the transcript. The nearest neighbour residues of the target C play a crucial role in editing, but the nature and position of the residue varies according to the editing site concerned. The target region seems to be formed by two regions 5′ and 3′, which can be separated by a maximum of two residues. Studies on single residue mutants concerning every position in the 23 nt region indicated that editing sites are affected differently by their neighbouring sequences. These results suggest that, notwithstanding the similar extent and location of *cis*-elements, the editing site recognition mechanisms may differ in plant mitochondria.**

## INTRODUCTION

The RNA editing process changes the coding properties of mRNAs in a very specific manner. This process involves a variety of genetic systems and occurs using different mechanisms [for details see (1) and references therein]. Like other maturation processes of the organellar transcripts, it is an essential post-transcriptional event in plant mitochondrial gene expression (2,3). The RNA editing process has important functional consequences since the nucleotide conversions usually alter the coding properties of the mRNA that is required for the synthesis of functional proteins (4).

In higher plants, the RNA editing processes in mitochondria and chloroplasts share many common features. In chloroplasts, the sequences flanking the target residue are involved in RNA editing, as demonstrated by *in vivo* studies in transplastomic plants (5,6). Similarly, in mitochondria, we previously reported data indicating that the *cis*-recognition signals defining an editing site were located in the immediate vicinity of the target C (7). In both organelles, the RNA editing proceeds by deamination of the C residue and the sequences surrounding the editing site exhibit no common characteristics (8–10). Moreover, a promiscuous chloroplast sequence is not edited in mitochondria (11); conversely, a mitochondrial sequence does not sustain editing when transcribed in chloroplasts (12). These results indicate that editing recognition signals in the transcripts are specific to each organelle.

The fact that sequences flanking target C residues do not share consensus elements at the primary or secondary structure level suggests that several hundred editing sites found in plant mitochondria (13) may require hundreds of specific factors. Alternatively, a few editing factors may recognize a subset of editing sites, as was speculated for chloroplasts. The overexpression of the region flanking an editing site inhibits the C-to-U modification of a group of editing sites in chloroplasts (14). To answer this question better, fine analysis of several editing sites is required.

Recently, Takenaka *et al*. (15) reported that the −15 to −5 region is essential for the editing reaction using a pea mitochondria *in vitro* assay. However, detailed study concerning the participation of the different residues that constitute the editing-site recognition element has not yet been performed. Here, for the first time, we present data comparing two editing sites of wheat cytochrome oxidase subunit 2 (*cox2*) transcript in an *in organello* system (7,16). To explore the function of the proximal region of editing site, we performed an extensive mutation analysis and monitored editing activity. We present evidence that the editing sites are accurately recognized when inserted elsewhere in the transcript, outside of their normal context. We found that the 5′ and 3′ regions involved in recognition require a minimal distance to direct editing. Finally, we demonstrate that although the mitochondrial *cis*-recognition elements of different editing-sites are similar in size, they have different recognition patterns.

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## MATERIALS AND METHODS

### Plasmids

All plasmids used in this study are derivatives of pCOX II (16). pCOX II contains 882 bp of the upstream region and 2009 bp of the coding sequence of *Triticum timopheevi* mitochondrial *cox2* gene (GenBank accession no. AF336134). The 3′ non-coding region contains a 533 bp insert from the terminator region of *T.timopheevi cob* gene (GenBank accession no. AF337547). A 23 bp sequence, inserted at position −60 of the promoter, and the *cob* terminator region provide specific sequences for isolating transgene transcripts by RT–PCR analysis with primers 1 and 2 (see below). The mutants were constructed with the QuickChange™ Site-Directed Mutagenesis kit (Stratagene) using the appropriate primers (see below) under the conditions indicated by the supplier. The complete sequence of the mutant *cox2* constructs was verified previous to use in electroporation experiments.

### PCR primers

(i) GCGGTGCAGTCATACAGATCTGC
(ii) TATCCAGATTTGGTACCAAAC

### Mutagenesis primers (only sense primers are indicated)

M68b: AATCGCTCTTTGTGA-(Δ10)-CCATGGCAATTAGGA
M68: CATGGCAATTAGGAA-(Δ16)-CTCAAGACGCAGCAA
MD: TATAGGATCTCAAGA-(Δ12)TATGATGCAAGGAAT
MB: ATGGCAATTAGGATC-(Δ5)-ACGCAGCAACACCTA
M2: ATGGCAATTAGGATC**A**CAAGACGCAGCAACA
M3: ATGGCAATTAGGATC**C**CAAGACGCAGCAACA
M4: ATGGCAATTAGGATC**G**CAAGACGCAGCAACA
77A: CCATGGCAATTAGGA**A**CTCAAGACGCAGCAA
77C: CCATGGCAATTAGGA**C**CTCAAGACGCAGCAA
77G: CCATGGCAATTAGGA**G**CTCAAGACGCAGCAA
M13: ACTATCGAAATTATTCCGGACCATATTTCCA
M40: ACTATCGAAATTATTCCCGGACCATATTTCCA
M40b: ACTATCGAAATTATTCCCCGGACCATATTTCCA
MK: ACTATCGAAATTATTTCGGACCATATTTCCA
ML: ACTATCGAAATTATTTTCGGACCATATTTCCA
MM: ACTATCGAAATTATTTTTCGGACCATATTTCCA
M56: CTATCGAAATTATTCGGGACCATATTTCCAA
MI: CTATCGAAATTATTCGGGGACCATATTTCCAA
MJ: CTATCGAAATTATTCGGGGGACCATATTTCCAA
MF: TACTATCGAAATTAT-(Δ1)-CCCGGACCATATTTCCA
MG: CTACTATCGAAATTA-(Δ2)-CCCGGACCATATTTCCA
MH: ACTACTATCGAAATT-(Δ3)-CCCGGACCATATTTCCA
M5: ATGGCAATTAGGATCCTCAAGACGCAGCAAC
M6: ATGGCAATTAGGATCCCTCAAGACGCAGCAAC
M7: ATGGCAATTAGGATCCCCTCAAGACGCAGCAAC
M8: ATGGCAATTAGGATC-(Δ1)-CAAGACGCAGCAACA
M9: CCATGGCAATTAGGA-(Δ1)-CTCAAGACGCAGCAA
AM: CAACACCTATGATGCAAGGAATCATCCATGGCAATTAGGATCTCAAGATGACTTACATCACGATATCTTTTTC
BM: ATACCATCGTTTGCTCTGTTATACTCCATGGCAATTAGGATCTCAAGACAATGGACGGGGTATTAGTAGATCC
CM: CAACACCTATGATGCAAGGAATCATTACTATCGAAATTATTCGGACCATGACTTACATCACGATATCTTTTTC
DM: ATACCATCGTTTGCTCTGTTATACTTACTATCGAAATTATTCGGACCACAATGGACGGGGTATTAGTAGATCC
MEb: ATGGCAATTAGGATCGGACCACGCAGCAACACCTAT
ME: CTCTTTGTGATGCTGCGGAATACTATCGAAATTATTCTCAAGACGCAGCAACACC

Inserted nucleotides are underlined, the modified residues are indicated in boldface and the number of deleted (Δ) residues is in parentheses.

### Purification and electroporation of wheat mitochondria

Electrotransfer experiments were carried out using purified embryo mitochondria corresponding to 1 mg of protein in 50 µl of 0.33 M sucrose and 1 µg of recombinant plasmid essentially as described by Farré and Araya (16). A 12 kV pulse was applied to the mitochondrial suspension contained in a 0.1 cm electrode gap cuvette. After electroporation, mitochondria were incubated for 18 h at 25°C in buffer containing 330 mM Mannitol, 90 mM KCl, 10 mM $MgCl_2$, 12 mM Tricine (pH 7.2), 5 mM $KH_2PO_4$, 1.2 mM EGTA, 1 mM GTP, 2 mM DTT, 2 mM ADP, 10 mM sodium succinate and 0.15 mM each CTP and UTP. After incubation, mitochondria were recovered by centrifugation at 15 000 *g* for 15 min at 4°C. RNA was purified with 200 µl of TRIzol™ reagent (Gibco-BRL) according to the supplier′s protocol.

### RT–PCR

An aliquot of 1 µg of RNA was treated with 2 U of Amplification grade DNase I (Promega). cDNA synthesis was performed with 200 U of Superscript II RT using 100 ng of random hexamers as proposed by the supplier. The PCR reactions were performed with primers 1 and 2 using Advantage® 2 Polymerase Mix (Clontech) as follows: 95°C for 1 min; 5 cycles at 95°C for 30 s and 68°C for 1 min; 30 cycles at 95°C for 30 s, 58°C for 30 s and 68°C for 30 s, and finally 68°C for 1 min.

### DNA sequencing and determination of editing rates

Sequencing of individual cDNA clones and mutant constructs was performed in automatic DNA sequencing equipment with the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems). To determine editing rates, sequence analyses were performed on the RT–PCR products using the Thermo Sequenase® radiolabeled terminator cycle sequencing Kit (Amersham). After electrophoresis, gels were dried and exposed to X-ray film. Autoradiographies were scanned using an UMAX Vista scanner and analysed with NIH Image software. The editing rate is displayed as a percentage of C-to-U conversion. Results obtained for each construct were highly reproducible showing variations below 5% between individual experiments. In all cases, the results presented correspond to a mean value of at least three independent electroporation experiments.

## RESULTS

All experiments were performed using a chimerical *cox2* gene, and mutant derivatives, controlled by its own promoter. The use of primers 1 and 2 (see Materials and Methods) allows specific amplification of the transgene products since the complementary sequences for these oligonucleotides are absent in the endogenous *cox2* gene and their transcripts. The RNA editing analyses were carried out on spliced products to avoid contamination with vector DNA. The mature *cox2* transcripts were sequenced in their entirety so that editing sites not involved in mutagenesis served as an internal standard. The editing efficiency observed for the wild-type transcripts is shown in Table 1. The results were obtained by sequencing 20 individual cDNA clones. With the exception of sites C482 and C638, which were not recognized, 15 out of 17 editing sites in the transcripts of the electroporated gene were edited in agreement with previous observations (16). Forty percent of the transcripts were edited at sites C30 and C682. Sites C77, C167 and C563 were found edited in 60% of the transcripts analysed. More efficient editing (80%) was observed for sites C169, C385, C587 and C620. All other editing sites were fully recognized. These results suggest that the editing machinery is able to distinguish each site in *cox2* transcript differently.

### A region that encompasses 16 nt upstream and 6 nt downstream defines the editing specificity of C77

Previously, the C259 editing site from *cox2* was found to be delimited by 16 nt upstream and 6 nt downstream of the target C (7). It is important to verify whether this rule is also valid for other sites. We observed that the 17 editing sites in *cox2* mRNA present different C-to-U modification levels. Indeed, while all *cox2* mature transcripts are edited at the C259 position, only 60% of the mRNAs contain a U residue at the C77 editing site, suggesting that the editing machinery recognizes each site differently. Therefore, we decided to focus our attention on the C77 site. The *cox2* transcripts issued from the exogenous construct, after electroporation of isolated mitochondria, presents ~60% editing efficiency. When the 16 nt upstream of C77 were deleted from this construct (mutant M68), edition was completely abolished (Figure 1A). In contrast, when the −17 to −26 region was deleted in mutant construct M68b, the residue C77 was edited at a higher rate, indicating that the upstream sequences, replacing the deleted ones provide a more favourable environment for recognition. The removal of five downstream residues inhibited the C-to-U change of C77 (mutant MB, Figure 1B). Also, the removal of residues +2 to + 6 abolished editing of C77 (data not shown). However, when the +7 to +18 region was deleted, RNA editing proceeds efficiently. These results show that the region comprising residues −16 and +6 is essential for C77 editing.

**Table 1.** Editing sites in the transcript of the *cox2* transgene

| Editing site number | | Editing rate (%) |
|---|---|---|
| 30 | CAUUAUCAUGUCGAUU**C**CUCACAAUCG | 40 |
| 77 | CCAUGGCAAUUAGGAU**C**UCAAGACGCA | 60 |
| 167 | AUUUUGGUUUUCGUAU**C**ACGGAUGUUG | 60 |
| 169 | UUUGGUUUUCGUAUCA**C**GGAUGUUGGU | 80 |
| 259 | UACUAUCGAAAUUAUU**C**GGACCAUAUU | 100 |
| 385 | UGGACAUCAAUGGUAU**C**GGACUUAUGA | 80 |
| 449 | ACUUUUGACAGUUAUA**C**GAUUCCAGAA | 100 |
| 466 | GAUUCCAGAAGAUGAU**C**AGAAUUGGG | 100 |
| 467 | AUUCCAGAAGAUGAUC**C**AGAAUUGGGU | 100 |
| 482 | CCAGAAUUGGGUCAAU**C**ACGUUUAUUA | 0 |
| 550 | ACGUAUGAUUGUAACA**C**CCGCUGAUGU | 100 |
| 563 | ACACCCGCUGAUGUAC**C**UCAUAGUUGG | 60 |
| 587 | UGGGCUGUACCUUCCU**C**AGGUGUCAAA | 80 |
| 620 | GCUGUACCUGGUCGUU**C**AAAUCUUACC | 80 |
| 638 | AAUCUUACCUCCAUCU**C**GGUACAACGA | 0 |
| 682 | UCAGUGCAGUGAGAUU**C**GUGGAACUAA | 40 |
| 704 | ACUAAUCAUGCCUUUA**C**GCCUAUCGUC | 100 |

A total of 16 nt upstream and 10 nt downstream of 17 target Cs (in boldface) are shown. The editing rate of each site was determined by analysing the sequence of 20 independent wild-type cDNA clones after electroporation of isolated mitochondria. The editing site number corresponds to the residue position in the *cox2* ORF considering the A residue of the initiation codon as the first base.

### 5′ neighbouring residue is important for C77 editing

We previously reported that the C259 downstream residue was important for editing (7). To test this possibility for C77, the 3′ U residue was changed for A, C or G. In all cases, the transcripts of the downstream mutants were edited (Figure 2A) although a reduction in editing efficiency was observed. These results strongly suggest that, contrary to the C259, the 3′ nearest neighbour nucleotide is not crucial for C77 editing. In contrast, when the 5′ U was changed to A or C, the editing efficiency was severely reduced, and completely abolished when changed to G (Figure 2B).

### The contribution of each nucleotide differs for each editing site

Site-directed mutagenesis was performed for every position in the −16 to +6 regions of C77 and C259 editing sites. To maximize the effect of the mutation on editing, the Cs and Us were changed in As and Gs respectively, and vice versa. Different patterns of editing effect were found for the two target sites (Figure 3A and B). While the changes at positions −1, −6, −9, −10 and −11 were responsible for reducing the efficiency of editing of the C77 site to <25%, changes at residues −11 and −12 had a strong inhibitory effect on RNA editing for the C259 site. Moreover, with the exception of residue +6, most changes at the six downstream residues from C77 have little effect; the downstream region of C259 presents three sensitive residues (+1, +3 and +4) in which modification significantly reduces the editing efficiency (Figure 3C).

### C77 is edited when present within a chimera that contains the 3′ region of C259

The *cox2* transgene was modified at site C77 by replacing either the upstream or downstream sequences by the homologous region from site C259. When the six 3′ residues of C259 editing site were located downstream of C77 (Figure 4, mutant MEb), editing proceeded but at low efficiency. In contrast, the C259 5′ region was unable to sustain editing when grafted upstream of the C77 site (mutant ME). Since the editing of the C259 site was dependent on the presence of a G residue at the nearest neighbour 3′ position (7), a C259/C77 chimeric site was constructed in which the 3′ neighbouring U residue of the C77 downstream region was replaced by G (data

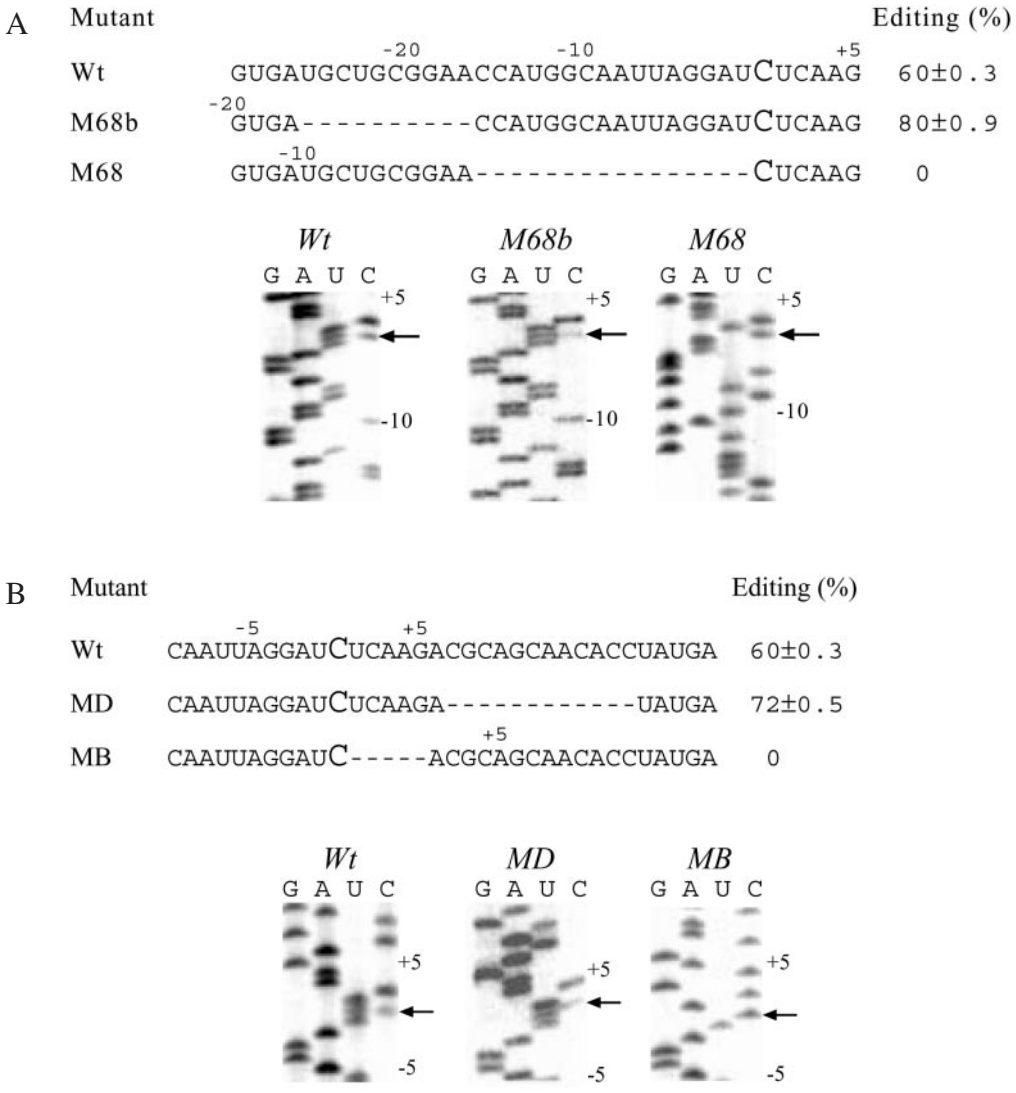


**Figure 1.** Deletion analysis in 5′ (**A**) and 3′ (**B**) of the C77 editing site. The deletions are indicated by dashes. The editing rates obtained from densitographic analysis of X-ray films are indicated. Target residue C77 is signalled in boldface and by arrows in autoradiographies. The results presented in this and the following figures correspond to a mean value of at least three independent experiments. In all cases, the variation observed between individual experiments was <5%.

not shown). This transcript was not edited, indicating that contribution of other nucleotides is required.

### The −16/+6 region is recognized when placed in a different context

Several mutants were constructed in which the −16 to + 6 regions of either the C77 or the C259 editing site were duplicated and were located in a different region of the first exon of *cox2* (Figure 5). Two constructs were tested for each site, one containing the additional site located at position 113, in between the wild-type sites C77 and C259 (Figure 5, mutants AM and CM), and other where the second site was located at position 322, downstream of C259 (Figure 5, mutants BM and DM). In all cases, the editing machinery recognized the grafted editing site as well as its wild-type counterpart with the same efficiency. At all the positions tested, the C77 and the C259 sites were edited in ∼60% and 100% of the mature RNA molecules, respectively. No difference in editing rate was observed for each site when placed at the normal or chimerical position. This observation clearly suggests that all the information required for recognition of editing sites is located in the immediate neighbourhood of the target C residue.

### Editing of C259 shows a 3′ dominance

Previously, we found that inserting an additional editable C residue, downstream of the target C259, does not affect the editing activity. However, the editing target was the added residue, C260 (7). We confirmed and extended this observation by inserting additional C residues downstream of the C259 editing site. A transcript with two added Cs (Figure 6A, construct M40) was edited at the residue C261 with low efficiency, indicating that the editing machinery recognized the cytidine closest to the six base 3′ domain. A construct with three added Cs (M40b) was not substrate for editing. Similar results were found when three U residues were inserted upstream of C259 (data not shown). When the added

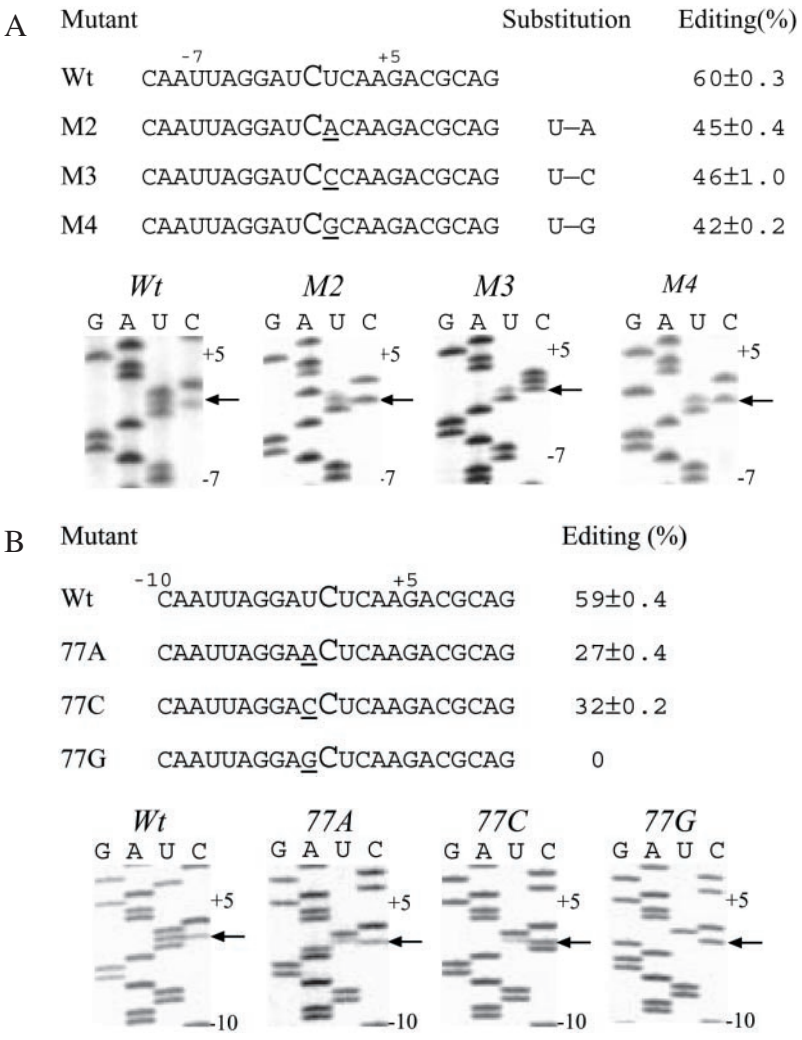


**Figure 2.** Analysis of substitution mutants at neighbouring nucleotides 3′ (**A**) and 5′ (**B**) of the C77 editing site. The changed bases are underlined. The editing position is signalled by arrows in sequence autoradiographies. Editing rates obtained from densitographic analysis of X-ray films are indicated.

residue was a G, a non-editable base, the ability to edit C259 was maintained (Figure 6B). The insertion of two G residues strongly reduced the editing efficiency, but the addition of three Gs abolished editing. This suggests that the 5′ and 3′ domains have to be close together to form an active editing site. To test this idea, the construct M40 (Figure 6C) was serially deleted from one (MF), two (MG) or three (MH) residues (A256, U257 and U258). All of them recovered high efficiency of editing.

### Editing of C77 shows a 5′ dominance

The addition of one, two or three C residues downstream of residue C77 induced a steady decrease in editing efficiency but did not abolish it (Figure 7A, constructs M5, M6 and M7). Unlike the situation observed for C259, the target residue remained linked to the 5′ domain as it was still C77. However, when either the 5′ or 3′ neighbouring nucleotide was deleted from the C77 site, editing was abolished (Figure 7B, constructs M8 and M9).

## DISCUSSION

The analysis of naturally occurring chimeric genes in the plant mitochondrial genome provides us with the opportunity to analyse a particular editing site in different contexts (17–19). Using this approach, Williams *et al*. (20) have suggested that 5′ flanking sequences may be crucial for editing-site recognition.

In this report, we define the regions required for the recognition of the specific C targets during the RNA editing process in higher plant mitochondria. Two sites were selected for further analysis, the residue C259, a very efficiently edited site, and the residue C77 which is edited in only 60% of the mature transcripts. The differences in editing rate might indicate some variations in the recognition mechanism of the editing machinery.

Previously, we found that the C259 editing site was defined by a short region spanning −16 nt upstream and no more than 6 nt downstream of the target C residue (7). We observed that a structure containing 16 nt upstream and 6 nt downstream could

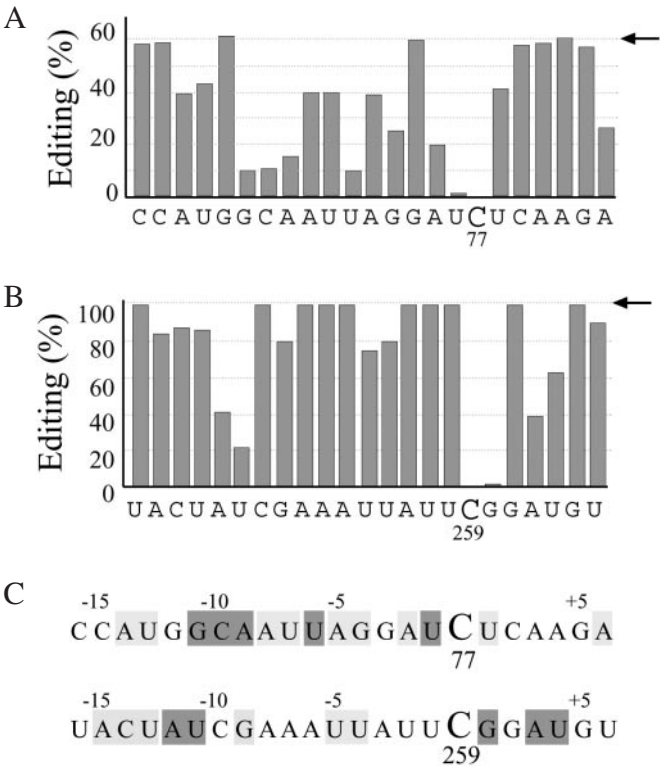


**Figure 3.** Point mutation effect on RNA editing at sites flanking C77 (**A**) and C259 (**B**). For each mutant, a single C or U residue was changed to A or G, respectively; A and G were changed to C and U, respectively. The arrows indicate the editing rates found in wild-type transcripts. The SD was <5%. (**C**) The residues strongly or moderately affecting the editing activity of C77 and C259 editing sites are indicated in grey and light grey, respectively. Editable C residue is in boldface.

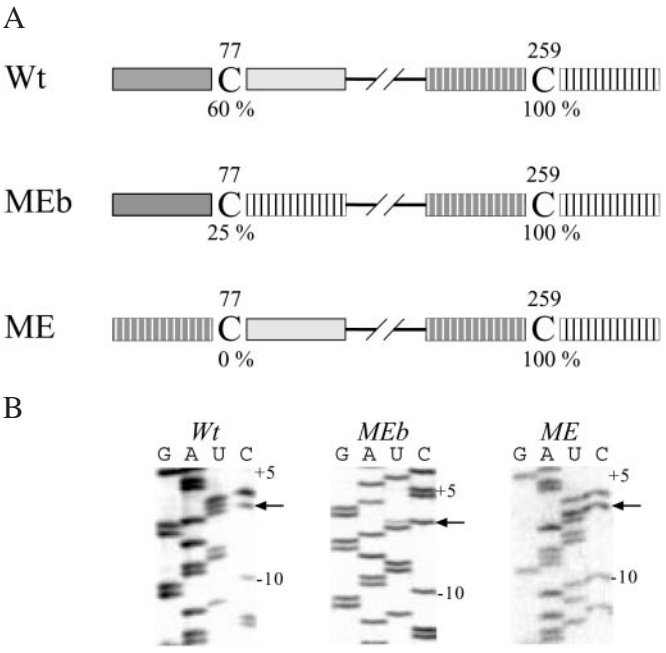


**Figure 4.** (**A**) Schematic diagram of chimerical editing sites obtained by replacing the 16 upstream residues or 6 downstream residues of site C77 with the 5′ and 3′ regions from C259. In all constructs, the C259 editing site remains unchanged. (**B**) Autoradiography of sequencing gels. Arrows indicate the target C residue.

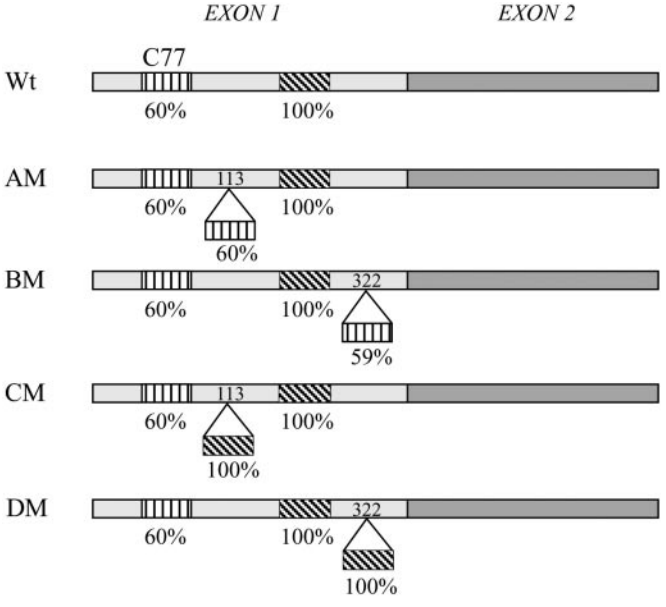


**Figure 5.** Scheme of *cox2* mutants presenting duplicated editing sites. AM and BM present the 23 nt corresponding to the C77 editing site at two different positions. The same situation is indicated for site C259 (mutants CM and DM). The editing rate, obtained from autoradiography film scanning, is expressed as percentage of C-to-U conversion.

also define the C77 editing site, suggesting that this region may be the minimal motif recognized by the RNA editing factors. This inference is supported by experiments in which fragments of 23 nt containing either C77 or C259 editing sites were located in different positions of the *cox2* construct. In all cases, both the wild-type and the translocated regions were accurately edited.

Analysis of the 17 editing sites in the *cox2* transcript does not reveal a consensus sequence that could give insight on the basis of the highly specific C-to-U changes in mitochondrial messengers (see Table 1). Indeed, when scrutiny is extended to the full set of 441 editing sites described in the *Arabidopsis thaliana* mitochondrial transcriptome, no clear consensus can be inferred (13). Our results clearly show that, inside the recognition 5′ and 3′ *cis*-elements, only some nucleotides play an important role. The G residue, immediately downstream of C259, was found to be mandatory for editing this site (7), but when we addressed this question to site C77, no such response was obtained. Changing the 3′ neighbouring U residue to a C, A or G, while reducing editing efficiency when compared to the wild-type sequence, does not abrogate editing. In contrast, changes at the 5′ neighbour residue reduce by 50% the C-to-U conversion when changed by A or C, and eliminated editing when changed to a G residue. This is a completely different situation from that described for C259 suggesting that the overall organization of editing sites is similar, but that the role of individual residues is different in recognition by the editing machinery. This is evident when comparing the response to single nucleotide mutants throughout the −16 to +6 *cis*-elements in both C77 and C259 editing sites (Figure 3C). The upstream −16 sequence

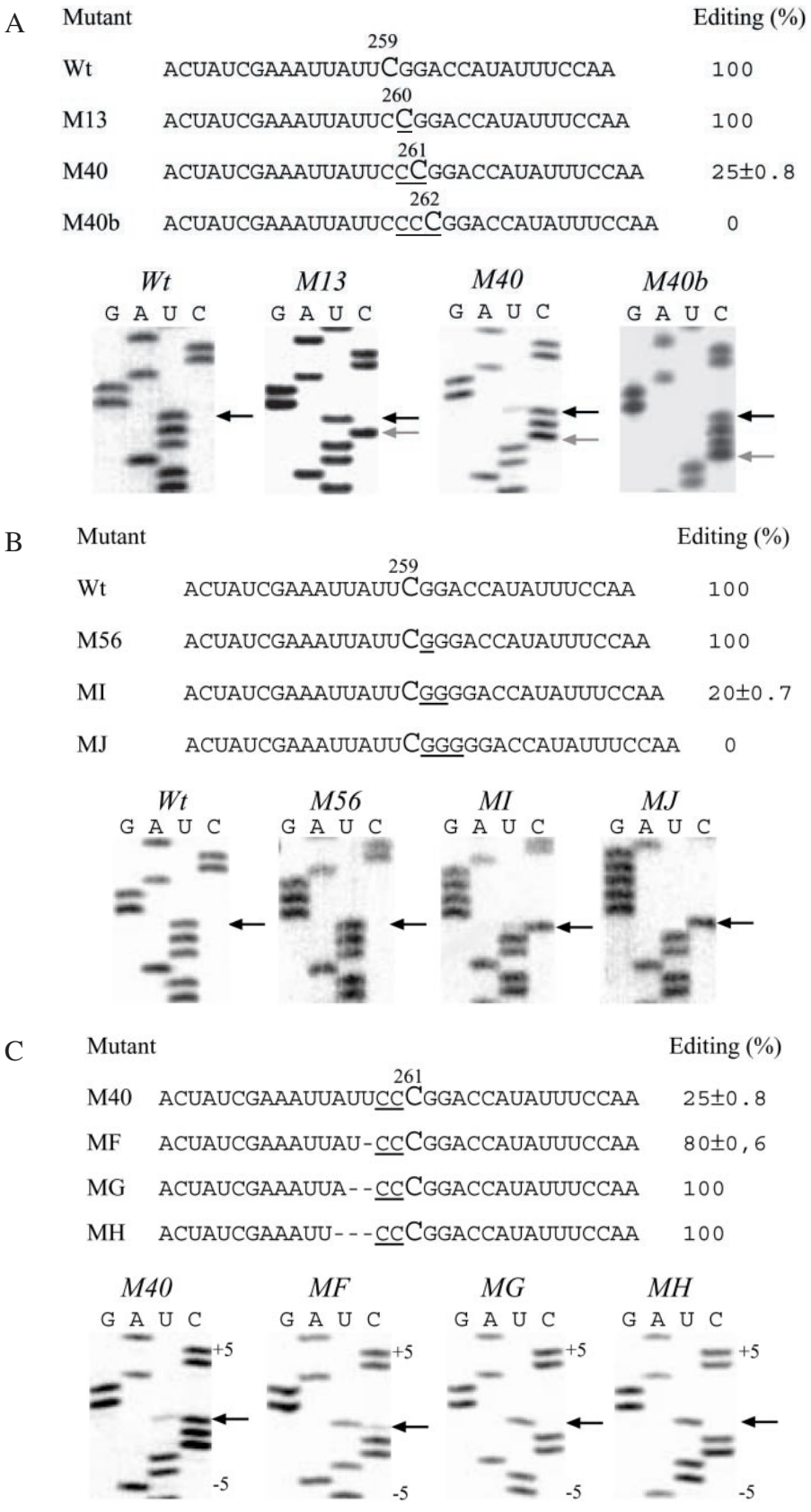


**Figure 6.** Analysis of insertion mutants on C259 editing site. (**A**) Insertion of one (M13), two (M40) and three (M40b) C residues at the 3′ position of C259. (**B**) Insertion of one (M56), two (MI) and three (MJ) G residues 3′ of C259. (**C**) Upstream deletion mutants from M40 construct. Editable C residue is in boldface. The inserted residues are underlined. Deletions are indicated by dashes. Editing rates obtained from autoradiography film scanning are indicated as percentages. Arrows indicate the edited C residue. Grey arrows signal the C259 position in insertion mutants.

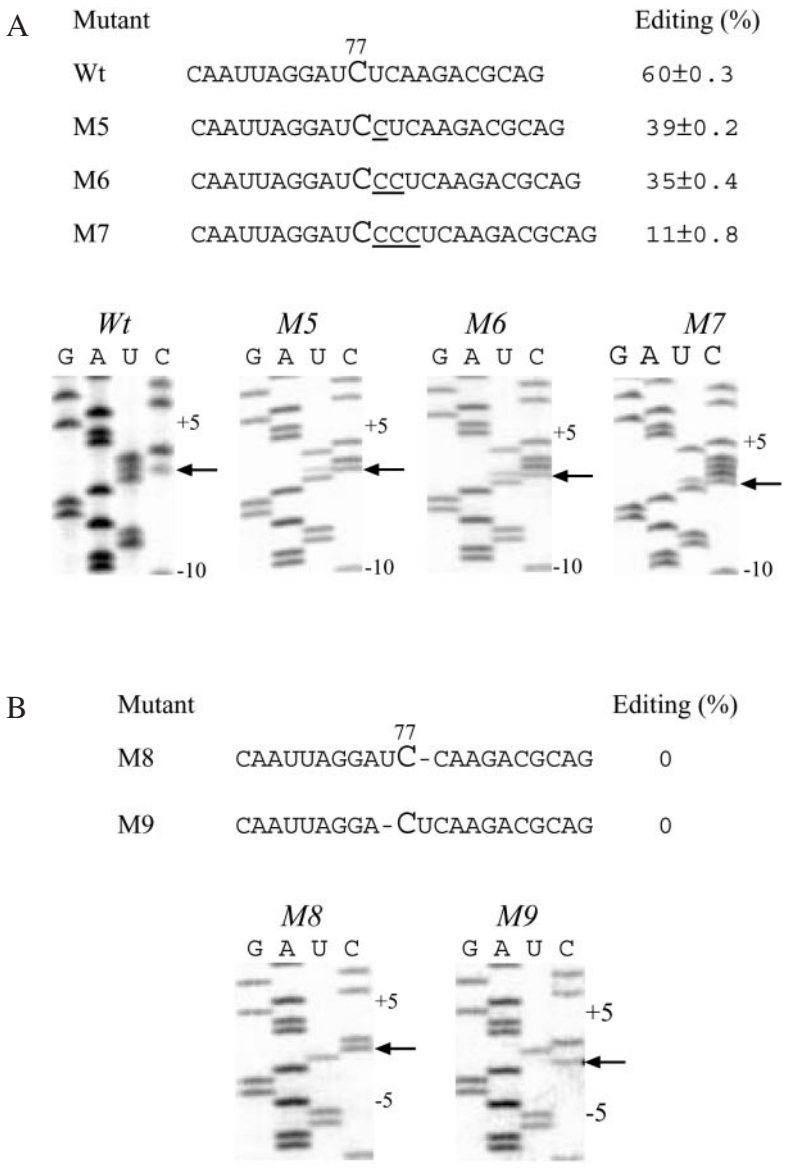


**Figure 7.** (**A**) Analysis of C insertion mutants of C77 editing site. (**B**) Analysis of 5′ and 3′ deletion mutants of site C77. The sequence and the editing level of each mutant are shown. Editing C residue is in boldface. The inserted residues are underlined. Editing rates obtained from autoradiography film scanning are indicated. Arrows indicate the target C residue.

of C77 was sensitive to changes with strong effects on mutations at positions −1, −6, −9, −10 and −11, and to a lesser extent other positions including downstream hexanucleotide. In contrast, C259 showed no strong dependence on the base nature at the −1 to −10 positions, but it is sensitive to changes at positions −11, −12, +1, +3 and +4. These results raise the question of the factors involved in editing-site recognition.

Using an *in vitro* approach, Takenaka *et al*. (15) found that the upstream −15 to −5 region was required for editing in pea *atp9*. While these results confirmed the role of the upstream region, they failed to show any significant contribution of the downstream sequence. Moreover, enhancer sequences were found at −40 to −35 and −25 to −19 in the *atp9* model. In contrast, we found an increased editing efficiency when some nucleotides were deleted either upstream or downstream of the core, −16 to +6, recognition region (see C77, mutants M68b and MD). However, no effects on editing efficiency were detected when the complete (−16 to +6) C77 or C259 editing sites were placed in different contexts in the *cox2* transcript suggesting that, notwithstanding some observed context effect, this situation may not represent an attribute shared by all editing sites. Some differences observed when using either *in organello* or *in vitro* approaches may be explained by the fact that the former involves the complete gene expression pathway and the latter confronts the preformed substrate with isolated *trans*-acting factors. In fact, during gene expression, transcripts have to interact in turn with different *trans*-acting elements, thus reducing the ability to fold in a putative defined stable secondary conformation.

The absence of consensus elements and the functional differences found for each individual residue described here are strong arguments for postulating a diversity of site-specific

recognition factors in editosome assembly. While gRNAs play a determining role in RNA editing in trypanosomatid mitochondria (21), no such molecules have been uncovered in plant mitochondria and chloroplasts (8,22). Our results clearly argue against the role of gRNA-like molecules since several mutations, which should destabilize the putative base-pairing structures characteristic of the interaction of gRNAs with the editing site, do not affect plant mitochondria RNA editing. Strong arguments have been reported for the role of specific proteins in editing-site recognition in chloroplasts where sequence-specific RNA-binding *trans*-acting factors are able to recognize different editing sites, suggesting that a set of *trans*-acting factors is involved in the recognition of editing sites (10,23,24). The results presented here indicate that a similar situation may indeed occur in plant mitochondria. However, several hundred editing targets are present in the mitochondrial transcriptome while only a limited number exists in chloroplasts: it is possible that the same *trans*-acting factors recognize several target sites. Since no bona fide *trans*-acting factor has been reported yet, further experimentation will be necessary to resolve this question. Recently, the pentatricopeptide repeat-containing protein family (PPR), which is important for organelle biogenesis, was proposed to account for the RNA editing *trans*-acting factors (25). However, the biological significance of these observations has yet to be elucidated.

Interestingly, the downstream hexanucleotide from C259, when replacing the 3′ region of C77, was able to sustain editing of this site at low efficiency. The upstream 16 nt sequence from C259 was unable to replace the C77 homologous region. This observation is consistent with the fact that the nature of individual residues at the 3′ of C77 does not seem to play a crucial role in editing. However, in spite of the differences on single mutant response found for both editing sites, the regions from both sides of the target C are required for correct recognition process. The C77 recognition mechanism is reminiscent of the situation found in chloroplasts where the major *cis*-acting recognition elements reside upstream of the editing site, whereas the 3′ flanking region provides only small quantitative contributions to the efficiency of editing (5,6,10,24,26). An important observation is the fact that a G residue at −1 position has a strong inhibitory effect on mitochondrial C77 and C259 *cox2* editing sites as found in two different editing sites in chloroplasts (24).

It is remarkable that when the up- and downstream sequences are separated by more than two additional nucleotides, editing is abolished or severely affected. This suggests that *trans*-recognition elements require a critical spatial configuration in the target sequence. This conclusion is strengthened by the results obtained with deletion mutants of construct M40 that reduce the distance between up- and downstream regions with a concomitant recovery of edition efficiency to the wild-type level. Moreover, the C259 editing target may be shifted to new positions when adding C residues, but resided at the normal position when non-editable residues are inserted. However, this does not seem to be the general situation since C77 maintains the position of the edited residue when adding supplementary cytosine residues. Taken together, our results indicate that editing recognition sites in plant mitochondria are confined to short sequences surrounding the editable C and that they are characterized by the ability to adapt, to a limited extent, to the editing machinery. While the upstream regions seem to play an important role in site recognition, downstream regions may be also required. The absence of a canonical structure in the editing sites of plant mitochondria transcripts might reflect the fact that distinct protein factors recognize each site.

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