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Complex *cis*-elements determine an RNA editing site in pea mitochondria

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ABSTRACT

The *cis*-requirements for the first editing site in the *atp9* mRNA from pea mitochondria were investigated in an *in vitro* RNA editing system. Template RNAs deleted 5′ of −20 are edited correctly, but with decreased efficiency. Deletions between −20 and the edited nucleotide abolish editing activity. Substitution of the sequences 3′ of the editing site has little effect, which suggests that the major determinants reside upstream. Stepwise mutated RNA sequences were used as templates or competitors that divide the *cis*-elements into several distinct regions. In the template RNAs, mutation of the sequence between −40 and −35 reduces the editing activity, while the region from −15 to −5 is essential for the editing reaction. In competition experiments the upstream region can be titrated, while the essential sequence near the editing site is largely resistant to excess competitor. This observation suggests that either one *trans*-factor attaches to these separate *cis*-regions with different affinities or two distinct *trans*-factors bind to these sequences, and one of which is present in limited amounts, wheras the other one is more abundant in the lysate.

INTRODUCTION

RNA editing in plant mitochondria alters >400 nt identities (1). In mosses and ferns both C to U and U to C changes occur, while flowering plants nearly exclusively alter C to U. In chloroplasts of vascular plants ~30–40 analogous editing events are observed, raising the possibility that similar if not the same activities act in both organelles (2–5). Comparisons between the RNA editing parameters in the two different compartments are needed to clarify this question.

In both organelles, the recently renewed efforts to develop *in vitro* assays for RNA editing (6,7) have yielded considerable progress by extending the information gained from the first investigation in plant mitochondria almost a decade ago (8,9). These prior experiments provided evidence that the biochemical reaction underlying the C to U change is most probably a deamination step, which does not cut the sugar-phosphate backbone of the RNA (8,9). It is presently unclear whether one of the classic deaminases, several of which have been identified in *Arabidopsis* (10), is involved, since zinc-ion chelators have no effect on the *in vitro* reaction (7).

Determinants of the specificity in mitochondria have recently been investigated for several editing sites in an electroporation assay (11–13). Sequence requirements for editing sites have been analyzed in chloroplasts in transgenic plastids (14–18) as well as *in vitro* (6,19,20). These assays confirmed and extended the conclusions previously drawn from rearranged sequences in mitochondrial genomes and the editing states of their transcripts (21), which had suggested that the sequences 5′ of the edited nucleotide are the main determinants of site recognition. The crucial *cis*-region usually extends ~20–30 nt upstream of the editing site. Exceptions have been documented for plastids, where 84 upstream nucleotides may not be enough to specify a given site (14). The sequence region downstream of an edited nucleotide seems to contribute little, in some instances <5 nt identities appear to have an influence on the identification of the editing site.

In chloroplasts, cross-linking experiments in an *in vitro* system identified different proteins to bind specifically to the upstream sequence regions (6,19,20). Most individual RNA editing sites show little or no discernible sequence similarity and consistent with this high RNA sequence variation distinct proteins are found to interact with different sites. However, similarities between upstream sequences of groups of several editing sites suggest that sequence-specific *trans*-factors may be involved that can recognize several sites (15,22). In addition, common protein factors may be involved in binding a larger number of such sites, since one or more of the cross-linking protein moieties show similarity to general chloroplast RNA-binding proteins by their apparent size and by their reaction with the respective antibodies (6,19,20). Furthermore several *trans*-acting factors appear to be limited in quantity, since *in vitro* competition experiments as well as analyses of transplastomic plants revealed diminished editing at the sites with sequence similarities in their immediate 5′ regions, suggesting the depletion of a necessary *trans*-factor (6,14–17).

To gain further information about editing site recognition in plant mitochondria, we have now analyzed the *cis*-elements at the first RNA editing site in the *atp9* mRNA in our *in vitro* system developed recently for RNA editing in pea mitochondria (7). Deletions and mutations distinguish distinct elements upstream of the editing site. Some of these are essential for correct recognition, while others enhance the efficiency of the reaction.

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MATERIALS AND METHODS

Preparation of pea mitochondrial extracts

Pea seedlings (*Pisum sativum* L., var) were grown at 24°C in the dark for 6 days. Mitochondria were prepared by differential centrifugation and purified on Percoll gradients as described previously (7). An aliquot of 400 mg of isolated mitochondria were lysed in 1200 µl extraction buffer (0.3 M HEPES-KOH, pH 7.7, 3 mM magnesium acetate, 2 M KCl and 2 mM DTT) containing 0.2% Triton X-100. After 30 min incubation on ice, the lysates were centrifuged at 22 000 *g* for 20 min. The supernatant was recovered and dialyzed against 5 × 100 ml dialysis buffer (30 mM HEPES-KOH, pH 7.7, 3 mM magnesium acetate, 45 mM potasium acetate, 30 mM ammonium acetate and 10% glycerol) for a total of 5 h. All steps were carried out at 4°C. The resulting extract (10–20 µg of protein/µl) was rapidly frozen in liquid nitrogen.

RNA substrates

DNA clones (patp9) were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing template RNA as described previously (7). Deletion clones were shortened by removing original mitochondrial sequences as indicated in the respective experiments. The outside bacterial anchors for the PCR amplification accordingly moved closer to the editing sites. Coincidental nucleotide similarities between these and the substituted mitochondrial sequences were taken into consideration when evaluating the nucleotide requirements for RNA editing.

In vitro RNA editing reactions

The *in vitro* RNA editing reactions were performed as described previously (7). After incubation, the template sequences were amplified using RT–PCR with one of the primers labeled with the Cy5 fluorophor. RNA editing activity was detected using mismatch analysis employing the thymine DNA glycosylase (TDG) enzyme activity (Trevigen). The TDG-treated fragments were separated and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham).

The efficiency of the *in vitro* RNA editing reaction was quantified by comparing the areas under the peaks of the cleaved and uncut DNA fragments. The ratio of the cleaved, i.e. edited, fragment to uncut DNA was used to determine relative efficiencies of the investigated conditions in each experiment. To allow comparisons and to determine the variation between individual experiments, the ratios of cleaved to uncleaved fragments were displayed as percentages of the standard reaction conditions.

Generation of mutant substrates

The 5′ deletion mutants were constructed using inverted PCR from patp9 with primers −40, −30, −20, −10 and −0, respectively on the one side and primer invertion1 on the other. The resulting fragments were digested with EcoRI to generate sticky ends in the primer contained EcoRI recognition site and were self-ligated. The 3′ deletion mutants were constructed using inverted PCR from clone atp9-30 with primer invertion2 and primers +10 and +0, respectively. The PCR fragments were digested with XbaI and self-ligated.

The mutant templates with defined sequence regions exchanged to their opposite sequence were constructed by introducing the respective complement pentanucleotide in primers M1–M10. PCR was performed on deletion clone atp9-40 with primer invertion1 and primers M1, M2, M3, M4 and M5, respectively, and in the second series with primer invertion2 and primers M6, M7, M8, M9 and M10, respectively. The resulting fragments were digested with EcoRI or XbaI, respectively, and self-ligated.

Competition assays

Wild-type competitor RNA was synthesized from the PCR product amplified with primers T7 and +10 from clone atp9-40. A complete plasmid-derived control RNA was synthesized from the PCR product amplified from pBluescriptIISK^+ with T7 and SK primers. The mutant competitors were synthesized from the PCR products amplified from clones M1 to M5 with the T7 primer and primer +10, and from clones M6, M7, M8, M9 and M10 with T7 and the respective mutant primers. One hundred attomoles of substrate and 1000 times (100 fmol) competitor RNA were first mixed and then incubated with the mitochondrial *in vitro* assay as described above.

RESULTS

Exploration of the 5′ *cis*-recognition region borders with deletion templates

The initial template tested *in vitro* contains 173 native mitochondrial nucleotides upstream and 49 original nucleotides downstream of the monitored RNA editing site in the pea mitochondrial *atp9* mRNA (Figure 1). To explore the limits of the necessary *cis*-sequence elements, we first tested a template containing 40 nt upstream of the editing site (−40 in Figure 2). This template was edited as efficiently as the original construct with 173 'native' upstream nucleotides (data not shown). We next constructed a series of deletion clones, in which the native sequences were removed in the steps of 10 nt up to the editing site (Figure 1B). Excision of the mitochondrial sequences in effect moves the 5′ plasmid sequences closer to the editing site. Since these replace the mitochondrial nucleotide identities, we took care to monitoring accidental sequence similarities (Figure 1B and discussed below).

In templates with only 30 nt conserved upstream of the editing site, RNA editing efficiency decreased to ∼50% (Figure 2A). Removal of the next 10 nt did not reduce the amount of editing further, which shows that the remaining 20 nt are sufficient to correctly identify the native editing site (−20 in Figure 2A). The comparatively high activity of the −20 deletion in comparison to the −30 deletion may be influenced by the chance similarity of 6 nt in the bacterial sequence with the sequence between −30 and −20 (Figure 1B, underlined sequences). Editing is completely lost when the next 10 nt upto −10, or all of the mitochondrial sequences are removed upstream of the edited nucleotide (−10 and 0 in Figure 2A).

This result suggests that the sequence arrangement of 20 nt upstream of the edited nucleotide is necessary and sufficient to

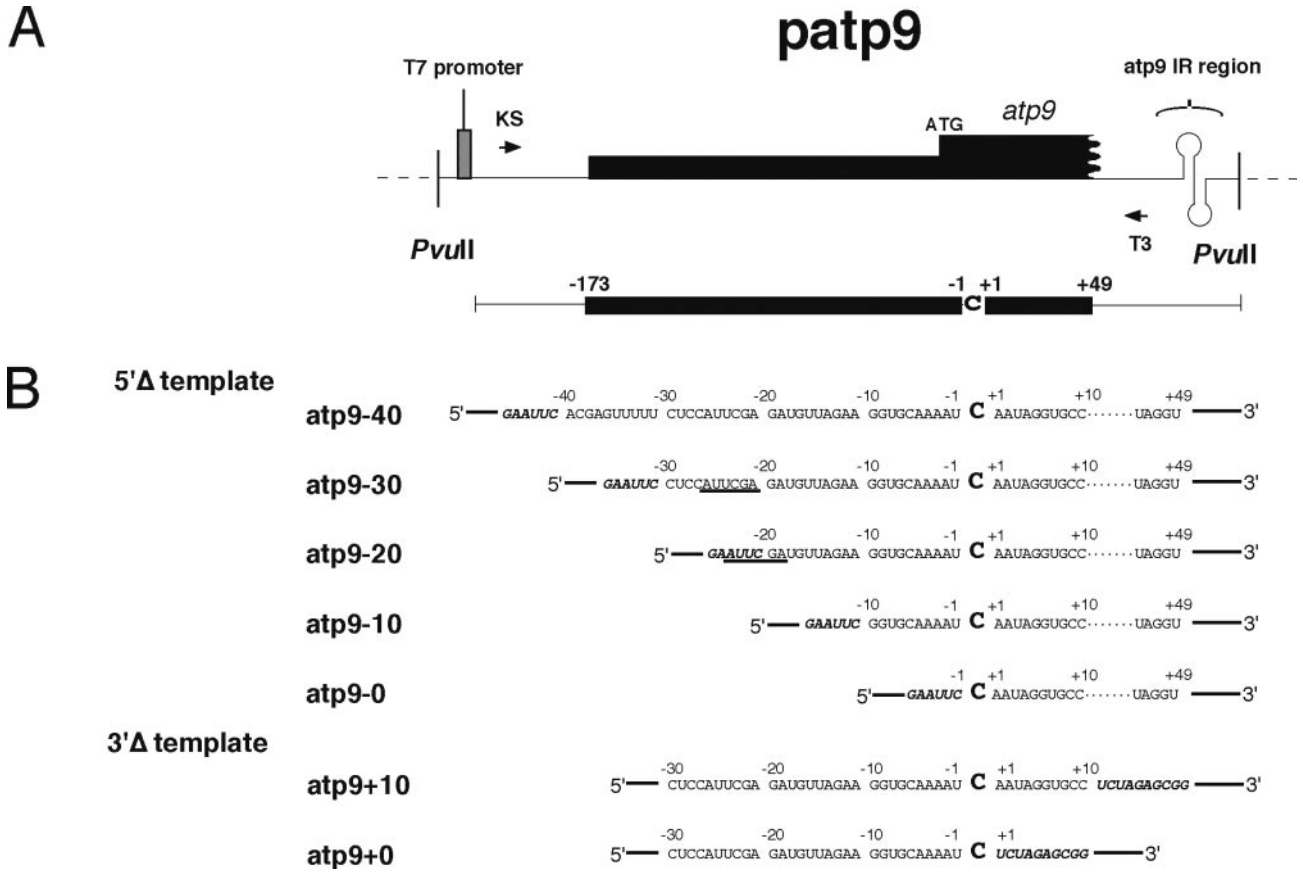

Figure 1. Structure of the RNA editing template and the construction of deletion clones. (**A**) The top line schematically depicts the plasmid embedded *atp9* gene fragment (bold black bar), off which the *in vitro* substrate is synthesized from the T7 promoter as a run-off RNA. In this RNA (shown in the line below), the *atp9* coding fragment is flanked by bacterial sequences and stabilized at the 3′ end by the *atp9* IR region (7). (**B**) Successively shortened templates were generated by deletions from the 5′ and 3′ ends, respectively. In these deletions, the excised *atp9* sequences move the primer binding bacterial regions closer to the targetted editing site. Each mutant respectively contains 40, 30, 20, 10 or 0 nt of the native *atp9* sequence upstream and 49 nt downstream of the editing site. A hexanucleotide sequence by chance identical between the deleted *atp9* sequence and the bacterial substitution (bold italics) in atp9-20 is underlined. The two 3′ deletion mutants tested include 30 nt upstream and 10 or 0 nt genuine *atp9* downstream sequence, respectively. The substituting bacterial sequence is given for 10 nt in bold italics. In the +0 clone the triplet UAG at positions +3 to +5 is incidentally present also in the bacterial sequence. The 5′ and 3′ black lines indicate the vector sequences containing KS and T3 promoters, respectively.

define the editing site and to identify the nucleotide to be altered. The upstream adjacent 20 nt between −40 and −20 contribute to an increase in the editing efficiency.

Charting the 3′ requirements with sequence deletions

The 3′ region downstream of the editing site was similarly investigated by templates processively deleted in the steps of 10 nt toward the edited nucleotide (Figure 3). In these constructs, the upstream region contained 30 nt identified as being sufficient to yield the accurate location of the editing reaction. To evaluate the sequence requirements in this region, three templates were tested containing +0, +10 and +49 nucleotides, respectively. All these deletions right up to the monitored C were edited correctly. Surprisingly, RNA editing became somewhat more efficient when more nucleotides were removed, i.e. substituted by bacterial sequences. This observation suggests that the *in vivo* sequence may be suboptimal at least in our *in vitro* assay and can be improved by changing some nucleotide identities.

From these experiments, we conclude that the minimal substrate region surrounding the editing site consists of 20 mitochondrial nucleotides upstream and no native nucleotides downstream of the edited C. Downstream sequences as well as the −40 to −20 upstream nucleotides, appear to modulate the *in vitro* editing reaction at this site.

Dissection of the requirements for editing site identification with mutated templates

To characterize the individual sequences necessary and/or supportive for editing of this site more in detail, we constructed mutants with consecutive sequence exchanges. In the steps of 5 nt, the native mitochondrial sequence was substituted by its respective antisense pendant (Figure 3A). The exchange of nucleotides −40 to −35 (M1) reduces the editing activity to <10% of the wild-type control, suggesting an important sequence element (Figure 3B). In contrast, the two exchanges between nucleotides −35 and −25 (M2 and M3) lower the editing efficiency only to 60 and 70%, respectively. The 10 nt between −25 and −15 upstream of the editing site are of comparable importance as the −40/−35 element, their alteration (M4 and M5) reduces the editing efficiency to ∼5–15%.

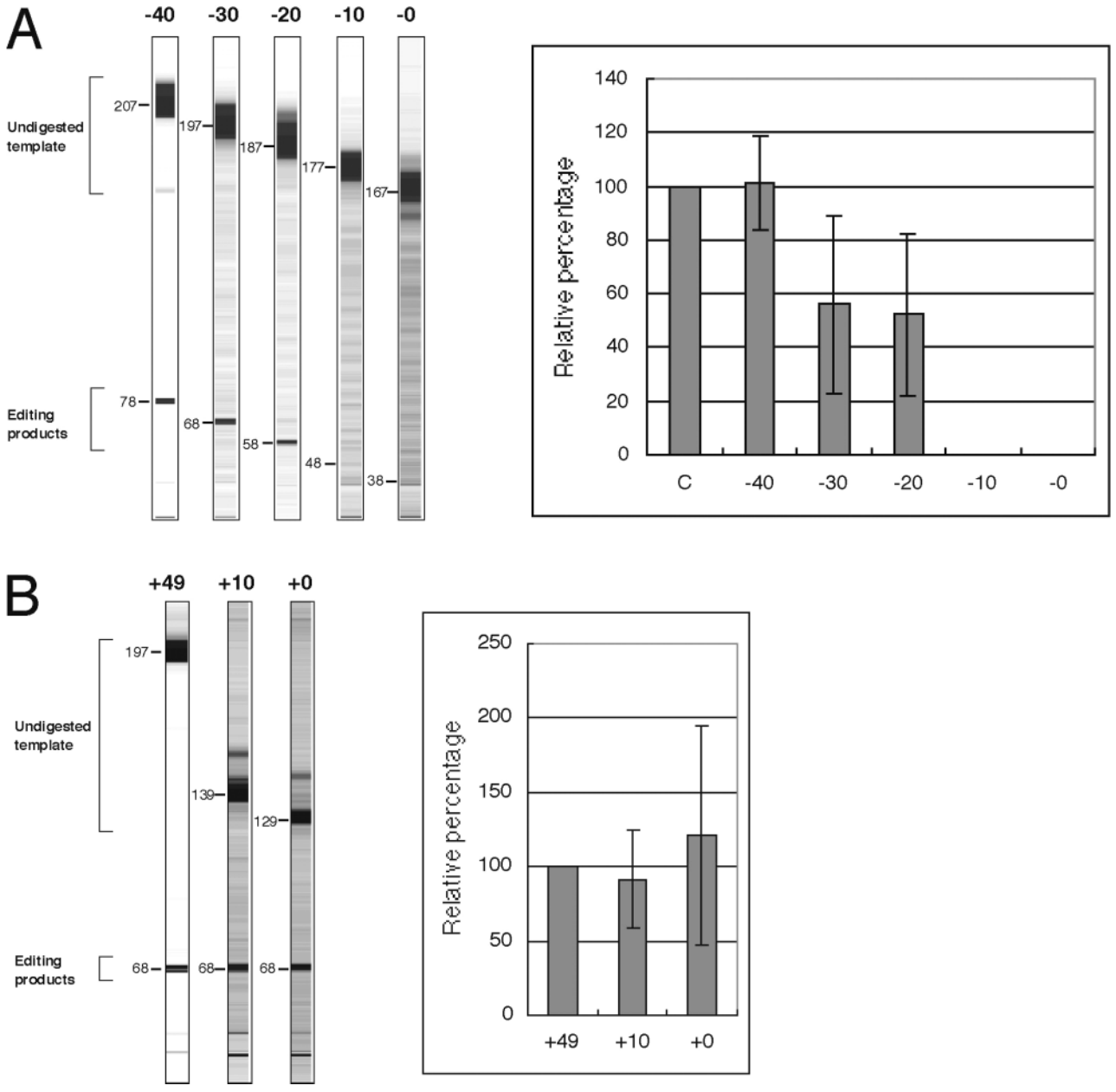

Figure 2. *In vitro* editing of deletion mutants shows 20 nt upstream to be sufficient to specify the editing site. (**A**) The 5′ deletion mutants reveal two levels of *cis*-sequences. The gel image of the TDG detection analysis is shown in the left panel. Sizes of unedited templates amplified as DNA fragments by RT–PCR between the Cy5 fluorescent dye labeled KS and the T3 primer and the predicted fragments resulting from cuts at the editing site are given in nucleotides for the respective deletion clones. Quantification of the respective editing efficiency (right panel) shows faithful editing with as little as 20 nt upstream of the edited C nucleotide. For full editing efficiency, however, 40 upstream nucleotides are required. In each of three experiments editing was quantified relative to the control template, the results were averaged and the standard deviation was calculated. (**B**) *In vitro* editing of 3′ deletion mutants suggests that the downstream nucleotides are probably not involved in marking the editing site. Gel image of a TDG analysis of the 3′ deletion mutants is shown on the left. DNA signals at 197, 139 and 129 nt correspond to the full-length RT–PCR fragments from atp9 + 49 (original clone length), atp9 + 10 and atp9 + 0, respectively, the DNA at 68 nt results from the fragments cleaved by TDG at the editing site. Quantification of the editing efficiency (right panel) shows correct editing even when all nucleotides downstream of the edited C nucleotide are substituted by bacterial sequences. Editing efficiencies are comparable in these deletion clones. The accidental sequence similarity and the high experimental variation have to be taken into account in the interpretation. In each of the five experiments editing was quantified relative to the control template, the results were averaged and the standard deviation was calculated.

Out of four assays with the mutant altered between −15 and −10 (M6) in different mitochondrial lysate preparations, we observed very low levels of editing and three times no editing only once. This result suggests that these nucleotides are crucially important, yet recognition and correct assignment of the nucleotide to be edited can occasionally occur without them. The sequence between nucleotides −10 and −5 was required absolutely to target this editing site, since we never see any editing in their respective mutant template (M7). The 5 nt immediately upstream of the edited C are less crucial, since

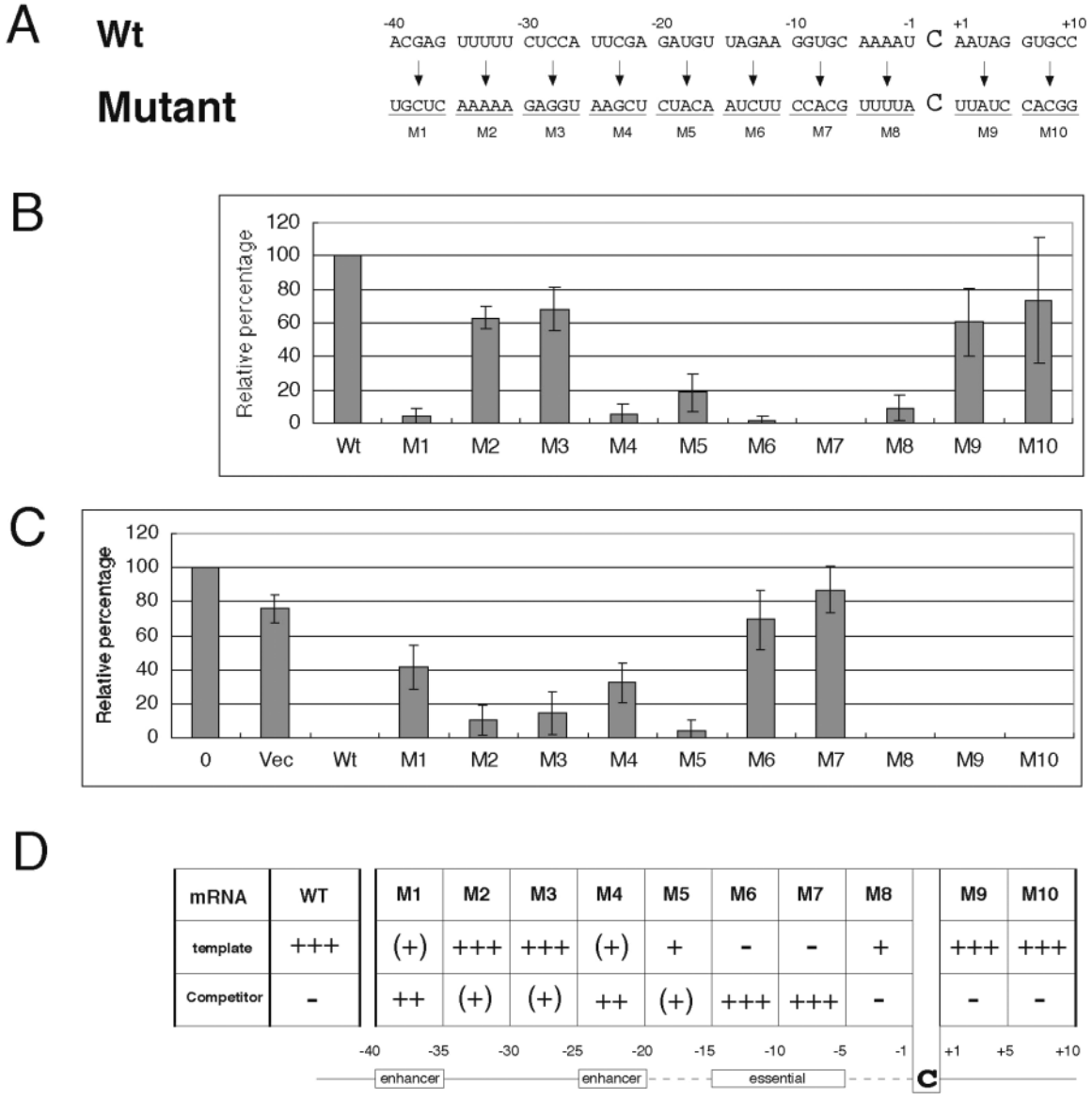

Figure 3. Mutant templates and competitors reveal distinct regions for recognition and efficiency of *in vitro* RNA editing. (**A**) Mutant *in vitro* editing templates/competitors were constructed by scanning mutagenesis with five consecutive nucleotides altered to their complement. The substituted sequence block is shown for each mutant construct M1–M10 underneath the wild-type sequence. (**B**) In each of four experiments, the respective editing efficiency was compared to editing of the co-analyzed wild-type sequence, the four results were averaged and the standard error was calculated. Mutant templates M1, M4 and M6 showed no clearly detectable editing in one or the other experiment, and yielded very little product in the respective other assays. (**C**) Mutant templates used as competitors further delineate the region upstream of the edited nucleotide sufficient for recognition of the template and for effective *in vitro* RNA editing. Mutant editing templates were added to the *in vitro* reaction in 1000-fold excess over the wild-type template. The respective editing efficiencies of three experiments were determined as the percentages of the editing of the wild-type sequence, the three results were averaged and the standard error was calculated. Mutant competitors M8, M9 and M10 showed no detectable editing in any of the assays, indicating that all essential and sufficient editing site determinants reside upstream of the edited nucleotide and can be completely titrated. (**D**) Comparison of the effects of mutant RNAs as templates and as competitors, respectively, delineates an essential recognition element and two sequence regions enhancing the reaction. The editing efficiencies in the individual experimental assays are categorized into full (+++), reduced (++), little (+), occasionally very little [(+)] and no detectable RNA editing activity (−). The complete lack of observed editing in mutants between nucleotide positions −15 and −5 defines an essential sequence region that cannot be substituted by any of the other surrounding sequence elements. In turn, these mutants have no discernible effect as competitors, suggesting that the other elements are recognized by distinct *trans*-acting factor(s) that are not titrated by the employed competitor concentration. Further details are discussed in the text.

RNA editing still occurs correctly in the respectively exchanged template RNA (M8), although with greatly reduced activity.

Mutations downstream of the editing site (M9 and M10) allow RNA editing to proceed at the correct nucleotide and affect the editing efficiency only mildly with a reduction of 30–40%, respectively, compared with the upstream mutations M2 and M3 (Figure 3B).

In summary, these assays with mutated templates resolve the initial classification of *cis*-regions by the deletion clones in more detail and allow tentative functional distinctions of the upstream elements. Two enhancing regions appear to be

located at −40/−35 and −25/−10, respectively, separated by less influential nucleotide positions. A promoting effect is also observed for the sequence immediately around the editing site itself. The essential recognition region of the to be edited nucleotide is located between −10 and −5 nucleotides upstream of the respective C, most probably stretching some nucleotides further upstream beyond the −10 position.

Competition with mutated templates suggests a limiting specificity factor

In the next series of experiments, the mutated RNAs are used as competitors of the wild-type template (Figure 3C). Competitors with mutations in non-essential regions will contain the wild-type versions of essential sequence elements and are thus expected to decrease or completely block editing in the monitored RNA. This is indeed observed: competition with mutations in non-essential regions M5, M8, M9 and M10 abolish detectable editing of the test substrate.

Conversely, mutant M7, in which the essential region between −10/−5 is eliminated, competes hardly at all with the template. Mutant M6 lacking the upstream adjacent region between −15/−10 similarly reduces editing only slightly. Surprisingly mutants M1–M4 inhibit the reaction to varying degrees, although all of them contain the essential region between −15 and −5. The different effects may reflect the individual contributing effects of these sequences observed with the mutants templates.

In control reactions the wild-type competitor completely blocks the reaction. The vector sequence alone reduces the editing process in the range of mutants M1 and M6, possibly due to some chance sequence similarities. These results confirm the distinction of separate *cis*-sequences, an essential region between −15 and −5, and enhancing regions at −40/−35 and −25/−15.

DISCUSSION

Here, we reported the analysis of *cis*-requirements for *in vitro* RNA editing in pea mitochondria that allows some functional conclusions about the recognition of an RNA editing site in plant mitochondria. Several *cis*-signals can be distinguished in the template by their respective influence on the reaction. This result suggests that either distinct protein (or RNA) molecules recognize these elements or one *trans*-factor attaches to different contact sites in the RNA.

The specific contribution of individual regions around (mostly upstream of) the investigated editing site are deduced from the relative *in vitro* editing reactions. However, we hesitate to interpret the precise percentages in these comparisons in fine details, since the variations between each individual experiment are in the range of the differences observed between individual constructs (compare the respective error bars in Figures 2 and 3). We thus restrict our interpretations to the five categories: full, reduced, little, occasionally very little and no detectable editing activity (Figure 3D).

All major determinants are located 5′ of the editing site

To investigate the extent of the *cis*-requirements, we tested the mutant templates processively deleted from the 5′ or 3′ terminus of the template (Figures 1 and 2). These deletions reveal an enhancer element in the region between −40/−30 and an essential recognition element between −20/−10. Deleting 3′ sequences has little influence on the overall editing activity, suggesting that no essential elements are located in this region. The somewhat enhanced reaction efficiency with complete substitution by bacterial sequences is hardly significant, but may be analogous to the observation made in a chloroplast RNA editing template (20), where the *in vivo* template may not be the optimal structure for (*in vitro*) editing. These results prove experimentally that the conclusions drawn from duplicated sequences in plant mitochondrial transcripts, where correct editing was observed when upstream sequences of ∼50 nt and downstream as little as 4 nt are conserved (e.g. 21).

An essential and two enhancing regions are separated by several nucleotides respectively

Mutation of nucleotides −40/−35 (M1 in Figure 3B and D) suppresses editing almost completely, suggesting that within these 5 nt important identities are located. Nucleotides downstream of this window from −35 to −25 before the editing site seem to be less involved in recognition. These little contributing 10 nt are followed by another important sequence between positions −25 and −15 (M4–M5). The competition experiments further define this region to be located mostly between −25 and −20 (Figure 3C and D; M4), because the mutant of this sequence M4 competes considerably less than the mutant of the downstream adjacent nucleotides M5. However, this sequence element probably does extend several nucleotides downstream, since this neighboring sequence appears to be important for recognition (mutant template M5 in Figure 3D), but not sufficient to compete for binding with the recognizing *trans*-factor (mutant competitor M5 in Figure 3D).

The adjacent less important nucleotides up to −15 lead up to the crucial recognition region around −10 (between −15 and −5). This essential region possibly reaches closer to the editing site into the −5 to −1 sequence, since mutation of this region (M8) drastically reduces its recognition as a template. However, as competitor this mutant M8 completely blocks the reaction, showing that these nucleotides are not sufficient to rescue recognition for the adjacent upstream element.

The sequences downstream of the editing site are clearly not relevant to define this editing site, since the respective mutants M9 and M10 show nearly wild-type levels of editing and as competitors fully suppress the editing reaction (Figure 4).

Intriguingly, the template mutated between −40 and −35 (which equals a −35 deletion clone) shows more than 90% reduction in editing (Figure 3D), while deletion up to −30 and even to −20 reduces editing only by ∼50%. This result may possibly suggest that the bacterial sequences moved up to −30 and −20, respectively, by chance contain nucleotides that can partially compensate for the missing −40/−35 sequence. Alternatively, the deletion up to −35 (caused by the inverted upstream pentanucleotide) and the further upstream bacterial sequences combine into an inhibitory sequence stretch or secondary structure, which lowers RNA editing activity. Such a chance similarity between bacterial and the template sequence in the −20 deletion clone (Figure 1B) may be the reason for its comparatively high activity (Figure 2A) and disguise the

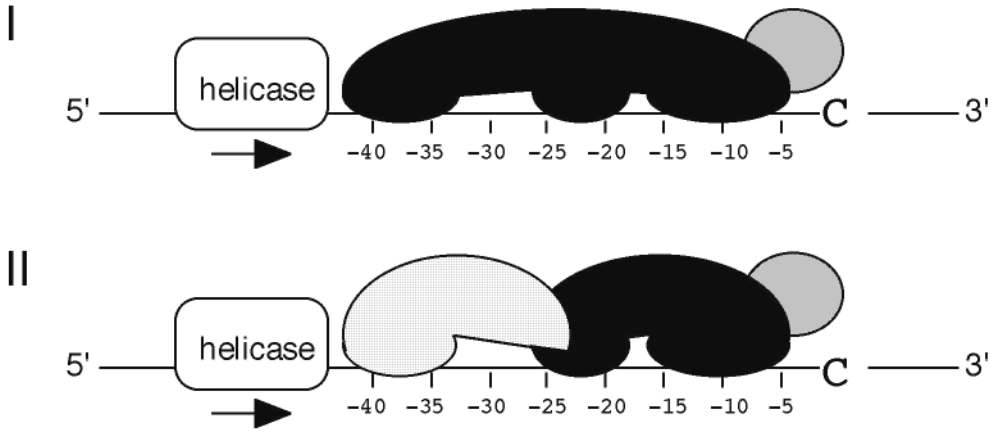

Figure 4. Models of *trans*-factors of RNA editing in plant mitochondria as deduced from the deletion, mutant and competition experiments are described in this report. The essential region and the two enhancing sequences are recognized by one *trans*-factor with distinct binding properties (model I; black body) or alternatively by at least two different *trans*-acting factors (model II; black factor and dotted cofactor). In both diagrams, the actual editing enzyme activity is drawn separately (hatched shape), but may also be an extension of the central *trans*-factor. The multiple coordinated factor model II is supported by the observation that binding at the essential region around −10 is titrated in the competition experiment, while the factor(s) binding to the upstream elements appear not to be out competed completely. The upstream elements between −40 and −35 and between −25 and −20 can each partially compensate the competition at the essential element between −15 and −5, suggesting that either two different *trans*-factors or one contacting these two binding sites can recruit and anchor the essential *trans*-factor binding at −15 to −5. A single *trans*-factor with distinct binding affinities at the three attachment regions could equally well explain the observations. The helicase either moves in to clear the RNA template before the editing complex binds or removes the complex after the actual editing is completed.

importance of the nucleotide identities between −25 and −20 (Figure 3B and D).

Distinct *trans*-factors in different stoichiometries or one factor with differential affinities?

The three separable *cis*-regions with different importance in the *in vitro* processivity of RNA editing suggest that each of them interacts differently with the *trans*-element(s) involved in identifying the site and catalyzing the reaction. This observation can be explained by two models equally consistent with the experimental information summarized here (Figure 4).

The first potential explanation for the observed behavior could be a single *trans*-acting (protein) factor, which extends its binding and recognition over the entire region between −40 and −5 nucleotide positions (Figure 4; model I). This *trans*-factor can contact different regions of the template, requiring the essential *cis*-element between −15 and −5 as major binding site. The non-essential enhancing elements then support this interaction and can partially substitute for the essential region. This model is supported by the experiment with mutant M1 as a competitor (Figure 3C and D): the observed (albeit lower) activity shows that this element may be able to partly substitute for −10 essential sequence. An analogous observation is made for the M2–M4 mutations, which can also partly rescue the competed essential element at −15/−5. These interpretations are of course only relevant if we exclude unpredicted structural effects of the deletions and mutations.

In the second alternative scenario, the specific reactions to individual competing sequence arrangements would be consistent with (at least) two different *trans*-factors contacting the RNA template at individual *cis*-elements (Figure 4; model II). The observation of little or no inhibition with the competing M6/M7 RNAs suggests that the two upstream elements at −40/−35 and at −25/−15 are recognized by *trans*-factor(s), which are not out-titrated by the 1000-fold excess of the respective added wild-type sequences. On the other hand, the putative *trans*-factor binding at −15/−5 seems to be present only in limited amounts, since it is only partially rescued by mutant competitors M1–M4 when compared to the complete inhibition of wild-type competitors.

In both scenarios, the NTP requirement may signal involvement of an RNA helicase as suggested from the previous biochemical analyses of *in vitro* RNA editing in pea mitochondria (7). The NTP requirement may be due to the initial binding of a *trans*-factor (protein), which the observed equally active dNTP substitution would suggest to be an RNA helicase. In this order of events, the RNA helicase would unwind and open secondary structures in the RNA template to allow firm contact with the other RNA editing complex proteins. The requirement for an RNA helicase as essential (co-)factor may however also signify a later step in the editing reaction, possibly the last, in which the attachment of a protein such as an RNA helicase may be required to dissociate the editing complex to allow its movement to the next site (23).

Similarities between RNA editing in plant mitochondria and chloroplasts

Here, the observed arrangement of various *cis*-elements for the first editing site in the *atp9* mRNA from pea mitochondria appears to be more complex than the *cis*-requirements of two chloroplast editing sites investigated in depth *in vitro* in the *psbE* and *petB* transcripts in tobacco and in pea, respectively (19). For the *psbE* editing site in tobacco (this site is not edited in pea), only one region between nucleotides −15 and −5 appears to be essential. Similarly, editing at the *petB* site requires one consecutive sequence stretch between −20 and −5, and possibly several downstream adjacent nucleotides in tobacco as well as in pea. This latter essential region, although larger than the *psbE* recognition site, is still only

half as large as the mitochondrial *atp9* editing recognition region identified here.

The differences observed between these *in vitro* results of site recognition in plastid and mitochondrial editing might reflect the differences between individual editing sites rather than a general interorganellar distinction, since mutational investigation of the editing site C259 in *cox2* transcripts in transfected mitochondria of wheat revealed a continuous specificity region covering only 22 nt from −16 to +6 around the edited nucleotide (11). For this site several downstream nucleotides appear to be essential, since complete substitution of the 3′ sequence even with that from another site did not recover activity. For another site investigated with this approach in wheat mitochondria, however, only upstream sequences appear to be necessary, analogous to the specificity requirements observed here for the *atp9* site in pea. These editing sites are thus specified by individually different *cis*-elements, which by extrapolation are recognized by unique *trans*-factors. Only sites with similar *cis*-sequence motifs could attract common factors, as observed in competition analyses in the transgenic chloroplasts (15,16).

For editing of the *psbE* site in chloroplasts, a two-step binding process for the identified p56 protein has been deduced from a detailed mutational analysis (20). The 56 kDa protein is proposed to bind initially upstream of the editing site and then to bend towards the C to be edited. For example, this second step may be hindered sterically by a G residue immediately upstream of the editing site. This process would be the equivalent of our single *trans*-factor model as depicted in Figure 4. Further experimentation is necessary to resolve these questions and to physically identify the *trans*-factors involved in RNA editing in plant chloroplasts and mitochondria.

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