RNA editing sites in plant mitochondria can share cis-elements

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Abstract RNA editing in flowering plant mitochondria alters numerous C nucleotides in a given mRNA molecule to U residues. To investigate whether neighbouring editing sites can influence each other we analyzed in vitro RNA editing of two sites spaced 30 nt apart. Deletion and competition experiments show that these two sites carry independent essential specificity determinants in the respective upstream 20–30 nucleotides. However, deletion of an upstream sequence region promoting editing of the upstream site concomitantly decreases RNA editing of the second site 50–70 nucleotides downstream. This result suggests that supporting cis-ltrans-interactions can be effective over larger distances and can affect more than one editing event.
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1. Introduction

RNA editing was first recognized in plant mitochondria and chloroplasts as a post-transcriptional process altering mostly C to U nucleotide identities in mRNAs and tRNAs. The more than 400 sites found in mitochondria in the mRNAs for only 53 genes imply that many sites are located relatively near each other [1]. This raises the question of whether these sites are addressed independently, collectively or consecutively.

Analysis of in vivo RNA editing in transgenic chloroplasts with individual gene fragments suggested that single sites can be edited faithfully, which is expected from their usually large distances from each other [2–6]. The development of reliable in vitro RNA editing activities for chloroplasts [7–9] and mitochondria [10,11] as well as in organello editing [12–15] in the past few years, has accelerated progress towards elucidating the cis-requirements. For plant mitochondria, in vitro RNA editing in pea lysates and in organello editing in wheat show that for some editing events only about 15–30 nucleotides are necessary upstream and very few or none downstream. These delineations of template requirements in a given mRNA template in plant mitochondria extend previous conclusions about the minimal recognition sequences from recombined transcript regions [16].

RNA editing on the template mRNA molecule appears to progress by site-by-site target recognition rather than a scanning process along the RNA molecule. This conclusion is based on the identification of cDNA clones edited at only some of the sites. The identity of these sites varies, which – supposing that these partially edited sites are editing intermediates – suggests that the editing activity attaches to the RNA molecules in numerous rounds at individual sites. Transfections of isolated mitochondria with cox2 gene sequences also yielded partially edited mRNA molecules, in which several sites are not edited in all or some RNA molecules [12–15].

To gather more direct data about editing site recognition we have now investigated whether neighbouring editing sites can influence each other. The interdependence of two sites in the apg9 mRNA separated by only 30 nucleotides was analyzed in an in vitro system from cauliflower mitochondria [17].

2. Materials and methods

2.1. Preparation of mitochondrial extracts

Heads of cauliflower were purchased at local markets. About 900 g of the top tissues of the inflorescences were harvested, manually chopped into small pieces and homogenized in a blender. Mitochondria were purified by differential centrifugation steps and a Percoll gradient [10]. Four-hundred milligrams of isolated mitochondria were lysed in 1200 μl extraction buffer [0.3 M HEPES-KOH, pH 7.7, 3 mM Mg-acetate, 2 M KCl and 2 mM dithiothreitol (DTT)] containing 0.2% Triton X-100. After 30 min incubation on ice, the lysate was centrifuged at 22000 × 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 Mg-acetate, 45 mM K-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 protein/μl) was rapidly frozen in liquid nitrogen. Mitochondrial lysates from pea seedlings (Pisum sativum L., var) were prepared as described [10].

2.2. RNA substrates

DNA clones (patp9) were constructed in an adapted pBluescript SK+ vector to allow run-off transcription of the editing template RNA as described [10]. Deletion clones were shortened by removing original mitochondrial sequences as indicated in the respective experiments. The outside bacterial anchors for PCR amplification accordingly moved closer to the editing sites. Coincidental nucleotide similarities between these and the substituted mitochondrial sequences as well as potential secondary structures were taken into consideration when evaluating nucleotide requirements for RNA editing.

2.3. In vitro RNA editing reactions

The in vitro RNA editing reactions were performed as described [10]. After incubation, template sequences were amplified by RT-PCR, the upstream primer labelled with the Cy5 fluorophor. RNA editing activity was detected by mismatch analysis employing the TDG enzyme activity (thymine DNA glycosylase, Trevigen). The TDG treated fragments were separated on 7.5% polyacrylamide gels containing 8 M urea at 900 V for about 400 min. The Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham).
The RNA editing product percentages lie well within the range of the linear sensitivity of the TDG assay as determined experimentally and the relative amounts of cut fragments (3–7%) thus reflect the amount of the in vitro editing activity within this window (about 1–25% [10]).

The efficiency of the in vitro RNA editing reaction was quantified by comparing the areas under the peaks of the cleaved and uncleaved DNA fragments. The ratio of cleaved, i.e., edited, fragment to uncleaved 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 results.

2.4. Generation of mutant substrates

The 5′ deletion mutants were constructed by inverted PCR from patp9 with primers –40, –20, –10 and –0, respectively, on the one side and primer inversion 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.

2.5. Competition assays

Wild type competitor RNA was synthesized from the PCR product amplified with primers T7 and +10 from the different patp9 deletion clones indicated in the figures. An entirely plasmid derived control RNA was synthesized from the PCR product amplified from pBluescriptIISK+ with T7 and SK primers. One hundred attomol of substrate and 1500 times (150 fmol) competitor RNA were first mixed and then incubated with the mitochondrial in vitro assay as described above.

3. Results

3.1. Detection of two adjacent editing sites in one template

In the plant patp9 mRNA the first editing site in the open reading frame is located 19 nucleotides downstream of the AUG codon. As depicted in Fig. 1A, this site is followed by a second editing event 30 nucleotides further downstream. The alignment shows that the respective upstream nucleotides of the two edited nucleotides, which for the first site have been determined to harbour the recognition region [11], show no primary sequence similarity. When a homologous template RNA containing both RNA editing sites and 40 nucleotides upstream of the first site (covering all cis-determinants for this site) is incubated with the mitochondrial lysate from cauliflower, the first and the second site are edited in vitro (Fig. 1B).

3.2. Editing of the second site depends on a species-specific template

In the plant patp9 mRNA the first editing site in the open reading frame is located close to the AUG and the upstream sequences in the 5′-UTR vary between species (Fig. 1A). To determine the influence of these species-specific sequences on the in vitro reaction we compared editing at the second site in template RNAs from cauliflower and pea, respectively (Fig. 2).

Surprisingly in vitro RNA editing with the heterologous pea patp9 template is much less efficient than with the cauliflower RNA: only in about one-third of the assays with the pea template is the second site observed with confidence, while the first site is consistently detected.

3.3. Thirty nucleotides determine the second RNA editing site

In the homologous cauliflower patp9 template the activity of the in vitro editing reaction at the second site is greatly diminished by deletion of the distant part of the first site recognition region, which harbours a sequence promoting editing at the

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**Fig. 1.** Two neighbouring RNA editing sites are processed in an in vitro lysate from cauliflower mitochondria (*Brassica oleracea*). (A) Nucleotide sequence alignment of the first two editing sites (1st and 2nd) in the patp9 mRNA reveals no sequence similarity around the sites. The edited C residues are shown by large bold letters, the upstream editing site (1st) of the top line pair is shown again in bold at the 0 position of the downstream site (2nd) in the lower part. In all experiments template RNAs are oriented and numbered relative to the upstream site, the first editing site in the mRNA. For comparison of the templates used and to see the divergent sequence in the region around –40/–35, the native cauliflower sequence (cf) is aligned with the pea (*Pisum sativum*) sequence and nucleotides identical between the two are marked by bullets. The AUG codon is framed. (B) A sample gel tracing of a cauliflower template RNA containing both editing sites. The enlarged graph shows the section of the scan covering the two editing sites. The second site is detected usually at about 20–25% of the efficiency of the first site. The respective gel image for this tracing is shown on the right, gel conditions were 7.5% polyacrylamide gels containing 8 M urea run at 900 V for about 400 min. Numbering on the left of the gel as well as underneath the scan gives the respective running times in the gel in minutes. Sizes of the RT-PCR fragments are given in nucleotides for the uncut and the editing site-specific products, respectively. Unspecific bands in the background appear optically more prominent in the gel image than in the actual scan. This background probably results from the RT-PCR, TDG and denaturing steps during the procedure.
first site (Fig. 2). Deletion of this element between nucleotides −40 and −20 relative to the first site results in a drop of the in vitro RNA editing efficiency at the second site by about 75%. A series of further deletion clones successively shortened in steps of 10 nucleotides from the 5′ end up to the first site has no further effect upon editing at the second site. The second editing site monitored here thus requires upstream only 30 nucleotides (or less) to sustain in vitro editing at a constant albeit low level. The heterologous pea template appears to contain editing–inhibitory sequences in the unique region between nucleotides −40 and −20 relative to the first site, since the deletion clones without this sequence are edited more efficiently (Fig. 2).

3.4. Only the cognate recognition sequences compete a given editing site

In vitro RNA editing at the first site is inhibited by competitor RNAs covering the first site sequences with the cis-recognition elements between −40 and +10 (Fig. 3B; −40/+10 cf). Downstream sequences as in the competitor −0/+49 have, as expected, little effect on the in vitro reaction, since the cis-elements for this site reside almost exclusively upstream [11].

The second editing site on the other hand is inhibited by competition with this latter −0/+49 RNA, further supporting the conclusion from the deletion templates described above that this region contains the cis-elements required for its recognition.

3.5. A cauliflower specific sequence supports editing over 70 nucleotides across the first editing site

To investigate whether the stimulating influence of the upstream element on in vitro editing of the second site is indeed species-specific, we first tested the effect of adding an excess of the pea upstream sequences covering nucleotides −40 to +10 relative to the first site (Fig. 3C; −40/+10 pea). The result of this experiment – no inhibition – allows three conclusions: Firstly, the pea distal element does not compete with the cauliflower sequence. Secondly, the cis-element of the second site does not seem to extend much further upstream beyond 20.
nucleotides, since the nucleotides 20–30 nucleotides upstream are included in this competitor and do not interfere. Thirdly, the first site recognition sequence has no detrimental effect on editing of the second site.

The enhancement of the reaction upon addition of the pea competitor −40/+10 (Fig. 3C) reflects an unspecific RNA effect, possibly by binding inhibitory non-specific RNA binding proteins, since a similar observation is made with unrelated RNA derived from vector sequences (Fig. 3C; sk). The observation of a possibly editing-inhibitory sequence in the unique region in the heterologous pea template between nucleotides −40 and −20 further supports the importance of this distant region for editing at the second site (Fig. 2).

3.6. The trans-factor addressing the cauliflower species-specific long distance supporting sequence motif seems to be abundant

In a further competition experiment the abundance of the species-specific trans-factor interacting with the distal upstream element supporting in vitro editing of the second site was investigated by adding excess homologous cauliflower sequences (Fig. 3C; −40/+10 cf). Editing of the second site was not influenced although this competitor greatly reduced in vitro editing of the first site (Fig. 3B; −40/+10 cf). The result that one but not the other can be competed, suggests that the trans-factors promoting editing of the first and second sites, respectively, from this same distal region act or are distinct.

4. Discussion

4.1. Editing sites are addressed individually

The template RNA constructs were designed to monitor two neighbouring RNA editing sites in order to determine whether access to these sites by the editing activity is connected or whether contact is made individually. Deletion templates containing only one or the respective other site show that either of the two sites can be edited in the absence of the other. The distinct recognition elements for the two sites are separated by about 5–10 nucleotides and presumably targeted individually by specific trans-factors.

This observation furthermore implies that there is no overt order in the alteration of the various nucleotides in a given mRNA. An apparent hierarchy might become established by the effectiveness of individual specific trans-factors to attract the hit-and-run editing complex and result in the observed partially edited mRNAs in the mitochondrial steady state population.

4.2. Specific trans-factors recognize neighbouring editing sites

The trans-factors attracting the RNA editing complex to the respective nucleotide to be edited are different for these neighbouring sites. There is no sequence similarity between the essential cis-regions which cover 23 nucleotides for the upstream and up to 30 nucleotides for the downstream site (Fig. 1A). Experimentally, the cross-competition experiments show that only the cognate sequence can interfere with editing at either site (Fig. 3). The different down-shifting with equal amounts of competing RNA molecules likewise supports this conclusion that the specificity factors for these two sites in the atp9 mRNA are distinct.

4.3. A species-specific editing-supporting sequence serves two sites

Efficient editing of the second site in the atp9 mRNA is observed only with the −40/+49 cauliflower template RNA in the cauliflower in vitro lysate, but not with the pea template and with neither template in the pea system (Fig. 2 and data not shown). This template includes the region at nucleotides −40/−35, which was previously identified to increase editing at the upstream site [17]. When this region is deleted or altered as in the pea template, efficiency of the reaction drops dramatically (Fig. 2). The native cauliflower template thus appears to attract a trans-factor which can act over a distance of 50–70 nucleotides to boost the editing activity at the downstream site. This cis-enhancer region can thus function for both neighbouring editing sites.

The effect is species-specific, since the different pea supportive region (Fig. 1A) cannot substitute for the positive effect of the cauliflower element for the second site. The pea enhancer region does however increase editing of the first site in the cauliflower in vitro system [17], suggesting that this pea sequence attracts in the cauliflower lysate (a) different (for the first site positive) trans-agent(s). That in the cauliflower template two distinct trans-factors may interact with the cognate cis-region, is further supported by the result that the positive effect of this region can be titrated for the first site, but not for the second site. The nature and identity of these trans-factors remain to be solved to determine the differential binding properties.

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