Abteilung Molekulare Botanik
(Leiter Prof. Dr. Axel Brennicke)
Universität Ulm

Recognition of a cluster of RNA editing sites in the *atp4* mRNA

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Daniil Verbitskiy

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Amtierender Dekan der Fakultät für Naturwissenschaften:
Prof. Dr. Klaus-Dieter Spindler

Erstgutachter:
Prof. Dr. Axel Brennicke, Abteilung Molekulare Botanik, Universität Ulm

Zweitgutachter:
Prof. Dr. Stefan Binder, Abteilung Molekulare Botanik, Universität Ulm

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1. Summary

RNA editing is the process of altering an RNA sequence from that encoded by the genome. In flowering plants organellar messenger RNAs are subject to C-to-U and less frequently U-to-C conversion. In most editing sites analysed *in vitro* or *in vivo*, sequences within approximately 30 nucleotides (nt) 5' and 10 nt 3' of the edited C have been found to be required and sufficient for selection of the correct C editing target and for editing efficiency. All previous studies focused on the investigation of processing of singular editing sites. Here I investigate recognition and processing of several editing sites located within few nucleotides in the mitochondrial *atp4* transcript. Here three editing sites are clustered in four nucleotides (**C**U**CC** at nucleotide positions 248, 250, 251). I find that a single *cis*-element of about 20 nucleotides serves for recognition of sites 1 and 3, while site 2 possibly requires a distinct recognition complex. Competition with this sequence element for sites 1 and 3 suppresses *in vitro* editing of both sites. Even RNA molecules in which all editing sites are substituted by A or G can compete *in vitro* editing of a wild type substrate indicating that editing site recognition can occur independently of the actual editing site**.** Experiments with partially pre-edited substrates confirms that the editing status of a substrate RNA does not affect the binding affinity of the specificity factor(s). *Cis*-element duplication within the RNA template dramatically increases the *in vitro* editing activity. This enhancing effect is observed when the distance between the editing sites of the individual repeats is about 25 nucleotides. If this distance is increased to 40 nucleotides the enhancing effect disappears. Analysis of the editing sites status in the repeat-containing RNAs shows a random distribution of the editing events between the repeats. This observation suggests a touch-and-go mechanism of RNA editing site recognition rather than a progressive linear processivity. *In vitro* experiments with two different editing sites with similar surrounding sequences suggest that such similar sequences can be recognized by the same trans-factor(s).

1. Zusammenfassung

RNA-Editing ist die Abänderung einer RNA-Sequenz von der im Genom kodierten Sequenz. In Blütenpflanzen sind die mRNAs der Organellen einer C-zu-U- und weniger häufig einer U-zu-C-Konversion unterworfen. Bei den meisten *in vitro* oder *in vivo* untersuchten Editingstellen zeigen sich Sequenzen mit ungefähr 30 Nukleotiden (nt) 5' und 10 nt 3' des editierten Cs als erforderlich und ausreichend für die Erkennung des zu editierenden Cs. Die bisherigen Studien konzentrierten sich auf die Untersuchung der Prozessierung einzelner Editingstellen. In dieser Arbeit wurden die Erkennung und Prozessierung von mehreren Editingstellen innerhalb weniger Nukleotide im mitochondrialen *atp*4-Transkript untersucht. Hier findet sich ein „Cluster" von drei Editingstellen innerhalb von vier Nukleotiden (**C**U**CC** in den Nukleotidpositionen 248, 250, 251). Es wurde festgestellt, dass ein einzelnes *cis*-Element von ca. 20 Nukleotiden der Erkennung der Stellen 1 und 3 dient, während die Stelle 2 möglicherweise einen anderen Erkennungskomplex benötigt. Wird dieses Sequenzelement in Konkurrenz für die Stellen 1 und 3 angeboten, so wird das *in vitro*-Editing beider Stellen unterdrückt. Sogar RNA-Moleküle, in denen die Cs alle Editingstellen durch A oder G ersetzt sind, können mit dem *in vitro*-Editing eines Wildtyp-Substrates konkurrieren, was zeigt, dass die *cis*-Elemente einer Editingstelle unabhängig vom Vorhandensein der tatsächlichen Editingstelle erkannt werden können. Experimente mit bereits teilweise editierten Substraten bestätigen, dass der Editingstatus eines RNA-Substrates die Bindeaffinität eines oder mehrerer spezifischer Faktoren nicht beeinflusst. Eine Verdoppelung des *cis*-Elements innerhalb des RNA-Substrates steigert die *in vitro*-Editingaktivität drastisch. Dieser verstärkende Effekt wurde beobachtet, wenn der Abstand zwischen den Editingstellen der einzelnen Sequenzwiederholungen ca. 25 Nukleotide beträgt. Wenn dieser Abstand auf 40 Nukleotide vergrößert wird, verschwindet der verstärkende Effekt. Eine Analyse des Status der Editingstellen in den Wiederholungen zeigt eine zufällige Verteilung der Editingereignisse zwischen den einzelnen Elementen. Diese Beobachtung deutet eher auf einen „touch-and-go"-Mechanismus für die Erkennung einer RNA-Editingstelle hin als auf eine fortschreitende lineare Prozessivität. *In vitro*-Experimente mit zwei verschiedenen Editingstellen mit einer ähnlichen umgebenden Sequenz zeigen, dass solche ähnlichen Sequenzen von einem oder mehreren gleichen Transfaktoren erkannt werden können.

2. Introduction

RNA editing is the process of altering an RNA sequence from that encoded by the genome. The phenomenon was first reported in the mitochondria of protozoa (Benne et al. 1986), in which insertion or, less frequently, deletion of U residues takes place in mitochondrial mRNAs. It was later discovered in the mitochondria (Covello and Gray 1989; Hiesel et al. 1989) and plastids (Hoch et al. 1991) of plants. In contrast to the protozoan system, organellar messenger RNAs are subject to C-to-U and less frequently U-to-C conversion in plants. In higher plants, RNA editing is necessary for the synthesis of functional organellar proteins (Begu et al. 1990; Marchfelder et al. 1996; Marechal-Drouard et al. 1996). While around 30 editing events have been described in the chloroplast transcriptome (Maier et al. 1995), more than 400 cytosines are changed to uridines in flowering plant mitochondrial transcripts (Giegé and Brennicke 1999; Notsu et al. 2002; Handa 2003). In contrast to the several hundred examples of editing events in plant or protozoan organelles, much fewer are known in mammalian mRNAs. For example: C-to-U editing in apolipoprotein B (apoB) mRNA generates a stop codon, resulting in translation of a shorter protein (Powell et al. 1987; Chen S.H. et al. 1987).

2.1. **Cis-***requirements in RNA editing site recognition in plant organellar RNA editing*

In plant mitochondria as well as in plastids, for most editing sites analysed *in vitro* or *in vivo*, sequences within approximately 30 nucleotides (nt) 5' and 10 nt 3' of the edited C have been found to be required for selection of the correct C editing target and for editing efficiency, while no consensus sequences have been identified. Using the tobacco plastid transformation system it was shown that a 22 nucleotides segment containing 16 nucleotides upstream and five nucleotides downstream of the editing site of the *psb*L gene is sufficient to direct efficient editing (Chaudhuri and Maliga 1996). A similar strategy on the *ndh*B editing sites showed that here the RNA segment from –12 to –2 is essential for editing (Bock et al. 1996). The *in vitro* system established for tobacco chloroplasts identified identical *cis*-elements for RNA editing (Hirose and Sugiura 2001).

The *cis*-sequences required for mitochondrial RNA editing were determined using *in organello* and *in vitro* systems. Using transformation of purified mitochondria, 16 nucleotides upstream and

6 nucleotides downstream were shown to be essential for editing of site 259 in the *coxII* mRNA (Farre et al. 2001). An in vitro system was established using a pea mitochondrial extract, with which for the *atp9* site 20 two regions were resolved, one from –40 to –35 which enhances efficient editing, and the second from –20 to –5, which is essential for the reaction (Takenaka et al. 2004).

These results suggest that the general features of the *cis*-elements required for RNA editing are similar in plastids and mitochondria. Generally, a less than 20 nucleotides upstream and 10 nucleotides downstream are sufficient for RNA editing. It is likely that these *cis*-elements are binding sites for *trans*-acting factors, which mediate access of the editing enzyme to the respective editing site.

2.2. Trans-*factors involved in RNA editing in the different systems*

Investigation of a chimeric RNA containing the editing site of *psbL* in tobacco chloroplasts had shown a significant decrease in the editing efficiency of the endogenous *psbL* RNA (Chaudhuri et al. 1995). This competitive effect of the trans-gene was specific to the *psbL* gene, with other endogenous sites being properly edited. This observation is strongly supported by *in vitro* analysis of *psbL* and *ndh*B mRNAs in the chloroplast *in vitro* system (Hirose and Sugiura 2001). The effect of competitors is specific to the corresponding editing site, suggesting that the *trans*-acting factor(s) is/are site-specific.

In the trypanosome system guide RNAs (gRNA) are involved in specifying editing sites. A pre-mRNA forms Watson-Crick base pairs and G/U base pairs with a gRNA, which identifies the site of RNA editing by complementary A or Gs. Endonucleotic cleavage of the pre-mRNA occurs upstream of the anchor duplex (8-10 bp) between the pre-mRNA and gRNA (Stuart et al. 2005). Uridines are either added or removed from the 3´end of the upstream pre-mRNA based on the sequence information present in the gRNA.

To investigate the potential involvement of trans-acting RNAs in RNA editing in plastids, the chloroplast extract was pre-treated with micrococcal nuclease. However, editing was still active after nuclease treatment (Hirose and Sugiura 2001). This observation eliminates the involvement of RNA factors in plastid RNA editing. Furthermore, ultraviolet (UV)-cross linking in the in vitro editing system suggests the involvement of protein factors in RNA editing in chloroplasts

(Miyamoto et al. 2002). Recently, it was reported that the pentatricopeptide repeat (PPR) protein CRR4 is essential for RNA editing of *ndh*D editing site 2 in chloroplasts (Kotera et al. 2005).

2.3. Incompletely edited mRNAs occur **in vivo** *in plant mitochondria*

In many editing events RNA editing is essential for expressing functional proteins, which are specified by the modified amino acid sequences (Sasaki et al. 2001), for generating a translation start codon (Hoch et al. 1991) or stop codon (Wintz and Hanson 1991). Therefore, many RNA editing sites have to be processed with high efficiency so as not to express mutant versions of proteins. However transcripts are edited with various degrees of efficiency. Analysis of more than 100 complementary DNAs (cDNAs) of the mitochondrial *nad3* gene in *Oenothera* failed to detect an RNA in which all the possible editing sites had been processed (Schuster et al. 1990). The most variable editing sites are usually in third-codon positions, where editing does not alter the corresponding amino acid (silent editing sites). However, even non-silent editing sites edited incompletely in these m RNAs, suggesting that the *nad3* gene may encode a series of proteins with minor sequence variations. However, in the similar situation of the *rps12* gene, both edited and unedited *rps12* transcripts are translated, but only translation products of fully edited mRNAs accumulate in mitochondrial ribosomes (Phreaner et al. 1996).

2.4. Biochemical processes and trans-factors of RNA editing

In the mammalian *apoB* mRNA editing system editing is implemented by a polysubunit protein complex. The APOBEC-1 (for "*apoB* editing catalytic subunit 1") protein is responsible for the editing reaction in the *apoB* mRNA. It contains a zinc-dependnt deaminase domain that is essential for cytidine deamination. Specific amino acids within the catalytic domain of the APOBEC-1 bind weakly to AU-rich sequences in the vicinity of the *apoB* mRNA editing site. This binding is insufficient for the specific RNA editing activity (Teng et al. 1993). Site specific editing requires APOBEC-1 homodimerization and an interaction with at least one or two other RNA-binding proteins ACF65 and ACF64 (for APOBEC-1 complementation factor). These proteins bind with high affinity to an RNA sequence element known as the mooring sequence located 3´of the edited cytidine, and together with APOBEC-1, form the minimal editosome (Mehta and Driscoll 2002). Each of the ACF proteins contains three RNA-recognition motifs

(RRM), these motifs are flanked by N-and C-terminal sequences that are essential for the ACF64/65 interaction with APOBEC-1 (Blanc et al. 2001). Thus, recognition of the *apoB* editing site is initiated by specific ACF protein-RNA interactions at the mooring sequence to form the required complex. Interaction of APOBEC-1 with this RNA-protein complex leads to alterations of the enzyme substrate specificity.

Presently much less is known about processing of editing sites in plant organelles. Several models have been proposed for plastid and mitochondrial RNA editing. Initially, using an in vitro editing system, mitochondrial editing was shown to be a deamination process of the specific C, rather than substitution or transglycosylation of the respective nucleotide (Yu and Schuster 1995). A similar enzymatic reaction has been proposed for plastids RNA editing (Hirose and Sugiura 2001). These results imply that a C deaminase is involved in RNA editing in plants, analogous to the APOBEC-1 in the mammalian system. It remains unclear if the enzymatic domain is part of a recognizing specific trans-acting factor or if it is a distinct protein. Since the CRR4 PPR protein reported to be involved in a specific chloroplast mRNA editing event does not contain any domains indicating a potential C deaminase activity, it seems more likely that a second factor is involved in this activity (Kotera et al. 2005).

The primary recognition of mitochondrial and plastid mRNA editing is also still unclear. From data obtained in the plastid *in vitro* system, the following recognition model was proposed for recognition of the *psbE* mRNA editing site in tobacco chloroplasts. In this model only one site-specific *trans*-acting factor binds an upstream *cis*-element in a sequence-specific manner (Miyamoto et al. 2004). However, the *in vitro* investigation of the *atp9* mRNA in pea mitochondria suggests that more than one recognition *trans*-factors may be involved in the recognition of this editing site (Takenaka et al. 2004). Moreover, inspection of the sequences surrounding chloroplast and mitochondrial editing sites reveals that they can be classified into groups which carry conserved nucleotides within 30 nt 5' of the C target of editing (Chateigner-Boutin and Hanson 2002). This observation and also the experiments with RNA editing of "foreign" constructs in non-cognate mitochondria (Choury and Araya 2006) suggest that one *trans*-acting factor may recognize several editing sites.

2.5. The aim of this work.

Previous studies had focused on investigations of singular editing sites, but in the mitochondrial transcriptome there are many examples of editing sites clustered within very few nucleotides. The recent *in vitro* analysis of neighbouring editing sites in the *atp9* mRNA separated by 30 nucleotides suggests that these sites can influence each other (van der Merwe et al. 2006). This work showed that these two sites carry independent essential specificity determinants in the respective upstream 20-30 nucleotides, that deletion of a sequence region promoting editing of the upstream site concomitantly decreases RNA editing of the second site 50-70 nucleotides downstream. These results suggest that neighbouring RNA editing sites in plant mitochondria can share *cis*-element.

How recognition of RNA editing sites separated by only very few nucleotides is however unclear. In this work I focus on the recognition of two RNA editing sites located within 4 nucleotides. To gain information about how such a cluster is addressed by the RNA editing machinery, I investigated *in vitro* the parameters involved in recognition of these sites.

3. Results

3.1. Selection of an editing cluster for in vitro *analysis*

Recognition of several mitochondrial and chloroplast editing sites has been analysed using *in vitro* and *in organello* systems. Most of these previous studies focused on single editing sites. However, there are also many examples of editing sites clustered within very few nucleotides in the mitochondrial genomes of higher plants. Recognition and identification of these editing clusters are so far unclear. Our global survey of *in vitro* editing competence of a number of mitochondrial genes in the cauliflower lysate showed editing activity for the cluster of editing sites at nucleotide positions 248, 250 and 251 of the *atp4* gene. To gain information about how such a cluster is addressed by the RNA editing machinery, I investigated the *in vitro* recognition of this cluster.

3.1.1. The atp4 *gene is altered at eight editing sites* in vivo

Genomic and cDNAs of the *B. oleracea* (cauliflower) *atp4* gene were cloned and sequenced (DQ202504). Sequence analysis showed that the cauliflower *atp4* gene has the same set of eight editing sites as the *A. thaliana atp4* gene (Fig. 3.1).

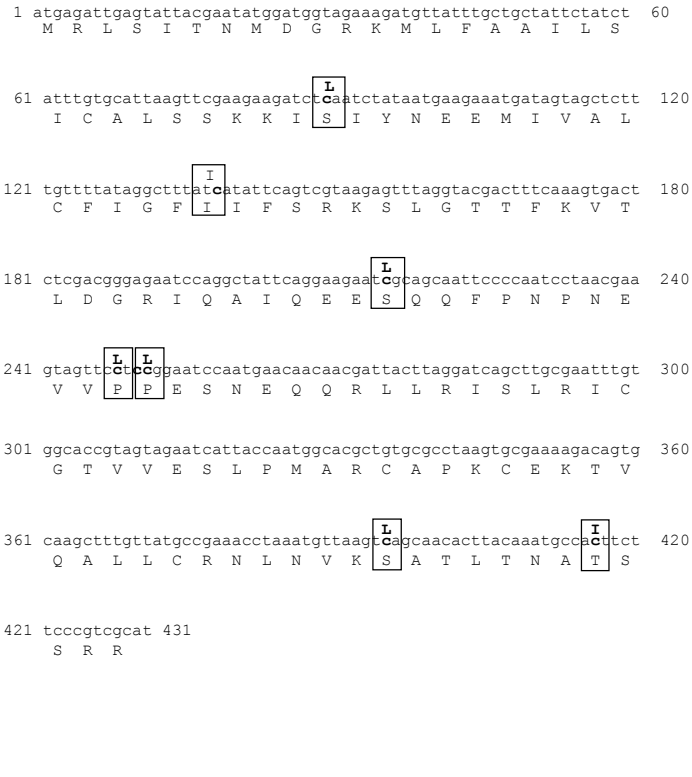


Figure 3.1. The nucleotide and amino acid sequences of the *B. oleracea atp4* gene. The editing sites and amino acid changes are framed. The editing sites at the nucleotide positions S89L, S215L, P248L, P250L, P251L, S395L, T416I cause amino acid alterations, but I138I is silent.

Most of the editing events lead to amino acids alterations, which enhance the number of hydrophobic residues in the protein, except for a synonymous change at nucleotide position 138 (counted from the A in the start codon). The editing sites at nucleotides 248, 250 and 251 (from here referred to as sites 1, 2 and 3) are clustered within 4 nucleotides of 2 adjacent triplets different from other sites that are distributed as singular sites along the gene sequence. *In vivo* the first and the third site appear to be slightly more rapidly edited, since of 38 cDNA clones sequenced, 37 are edited at all three sites, while one clone is edited only at sites one and three. This observation, although statistically not significant, nevertheless suggests that *in vivo* site two is edited somewhat slower than the other two. In terms of information content, site two is a silent editing as soon as site three has been altered and mRNAs unedited or edited at site two specify the same protein (Figure 3.2. a). Looking at other plant species, most, including dicots, monocots and gymnosperms, require editing at site one, while only dicots code C residues to be edited also at sites two and three (Figure 3.2. b).

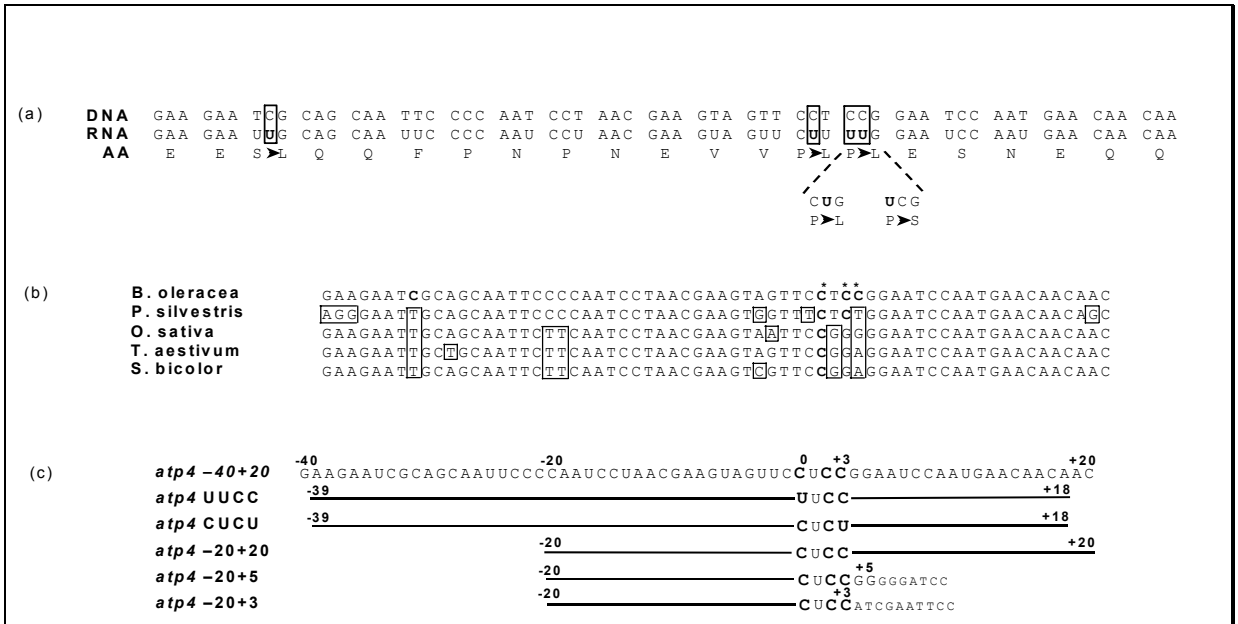


Figure 3.2. Three RNA editing sites are clustered within four nucleotides in the *atp4* coding sequence.

(a) Effect of RNA editing at the clustered sites on the encoded amino acid sequence. After editing of the third site the second site becomes a silent editing event, which does not change the amino acid specified. Uridines created by RNA editing are shown in bold and the resulting amino acid (AA) changes are indicated.

(b) Dicot plant species such as cauliflower (*B. oleracea*) contain a cluster of three editing sites, while monocots (*O. sativa*, *T. aestivum*, *S. bicolor*) and gymnosperms (*P. silvestris*) require editing only at the first site. In the latter species editing at this site has not been experimentally shown. Nucleotide divergencies from the cauliflower sequence are boxed. The cluster of the three editing events in cauliflower is marked by asterices. An additional upstream RNA editing event in cauliflower is indicated in bold and the consensus T at this position in other species is boxed.

(c) The initial substrate subcloned from the full length gene in cauliflower mitochondria contains 40 nucleotides upstream of the first edited C in the cluster and 20 nucleotides downstream (atp4 –40+20; top line). Nucleotide positions are all numbered relative to the first editing site in the cluster, which is assigned position 0. Sequences of partially pre-edited RNA substrates as well as various deleted substrates examined for *in vitro* editing are depicted in the lines below. The three C-nucleotides edited *in vivo* as well as the respective edited U-moieties are shown in bold and larger type. Smaller fonts in the -20+5 and -20+3 deletion clones give the substituting bacterial sequences for comparison.

3.1.2. In vitro *the first and the third of the three sites are edited*

In the cauliflower *in vitro* editing system sites one and three are altered correctly. The second site is not edited *in vitro* in line with the *in vivo* observation that this site may be somewhat less accessible to the editing machinery. To investigate the correlation of the editing events the individual clones derived from the *in vitro* PCR product were sequenced. Out of 20 analysed clones three were edited at both sites, while two were altered only at site one, and one was edited only at site three. This observation suggests, that there is no hierarchy in the editing events at sites 1 and 3.

3.1.2. Incompletely edited substrates are recognized as well as unedited sequences

To investigate the influence of the processing status of individual editing sites on recognition of this region I constructed substrates in which the first and third sites were "pre-edited", i. e. already contained uridines at the respective positions. Both substrates are edited at the residual site with efficiencies comparable to the completely unedited substrate (Figure 3.3). The RNA template with cytidine only at the second editing site did not show *in vitro* editing activity. This observation possibly suggests that this editing site is addressed by a different recognition complex.



Figure 3.3. Analysis of *in vitro* RNA editing in unedited and partially pre-edited substrates. The influence of processed versus unprocessed editing sites on the editing efficiency at respective other sites targetted by the same cis-element is investigated in a comparison of editing in an unedited mRNA substrate (left lane, atp4 **C**U**CC**–40+20), in an RNA edited at site 1 (center lane, atp4 **U**U**CC**-39+18) and in a substrate edited at site 3 (right hand lane, atp4 **C**U**C**U-39+18). The signal of the third editing site in the completely unedited substrate appears to be weaker due to the action of the TDG enzyme, which in this majority of doubly edited products will render the downstream site undetectable when having cut at the first site. The unedited substrate RNA covers 61 *atp4* nucleotides, each of the partially pre-edited substrates 58 nucleotides respectively. The TDG products of the latter substrate RNAs are 1 nucleotide shorter than the product from the unedited substrate, hence the corresponding shift in mobility. In the *in vitro* reaction of course only the respective residual not pre-edited site of the two potentially processed editing sites within this *atp4* region can be monitored. Sites of *in vivo* RNA editing are highlighted in bold as unedited **C** as well as editing product **U**.

3.1.3. Twenty nucleotides upstream of the first site and two nucleotides downstream of the last editing site are sufficient for **in vitro** *recognition of both editing targets*

In vitro analysis with various deletion substrates (Figure 3.2 c) reveals that upstream of the first site only twenty nucleotides are required for correct editing at both sites (Figure 3.4). The efficiency of editing in this RNA is however lower than with a substrate containing 40 nucleotides upstream. This result suggests that the sequence context further upstream of the immediate 20 nucleotides supports the attraction of the trans-acting editing protein(s), but is not essential.

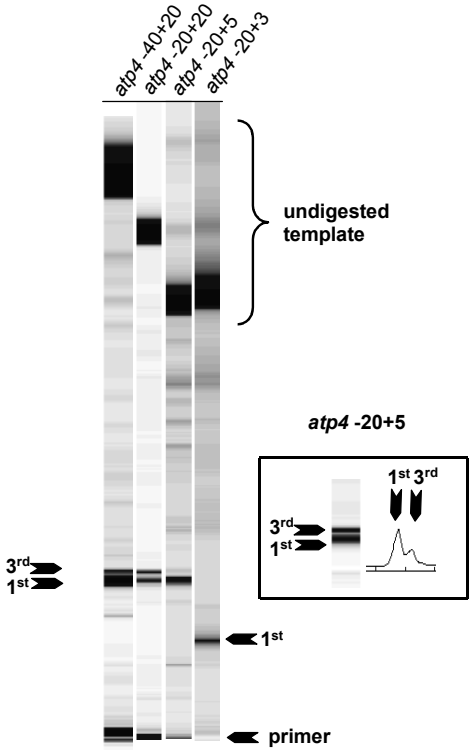


Figure 3.4. Determination of the minimal recognition site for the *atp4* editing site cluster. The longest substrate shown here (left lane, -40+20), covering 40 nucleotides upstream and 20 downstream of the first editing site, is edited rather efficiently at both the first and third sites. The 5'-deleted substrate –20+20 with only 20 upstream nucleotides shows diminished editing activity at site 1. Deletion of downstream sequences in substrate –20+5 with only 2 nucleotides conserved downstream of the last site reduces the level of editing at both sites. While only little *in vitro* editing product is seen at site three, the site as such is still recognized correctly. The inlet shows the gel image as well as the scan of a separate experiment with the –20+5 construct, in which the third site is clearer discernible. This 3'-most site is however not edited at all when the downstream adjacent nucleotides are substituted by different nucleotides in the -20+3 template (Figure 1c). The –40+20 and the –20+3 constructs are cloned in the SmaI site of the vector and the –20+3 TDG product is accordingly 20 nucleotides shorter. The –20+20 and –20+5 fragments are cloned into the EcoRV site of the vector, which is 18 nt distant from the SmaI site. This results in TDG products with similar 5'-sequence extensions.

In the downstream region five nucleotides beyond the first editing site (i. e. two nucleotides beyond the 3'-terminal site) are sufficient for effective editing at both target nucleotides. Removal of further two nucleotide identities does not inhibit editing at site one, but abolishes alteration at the 3'-terminal site three, suggesting that these downstream moieties are necessary for this latter event. In the substrate these two nucleotides were not actually deleted, but the GG dinucleotide was substituted by AT residues from the vector. I thus conclude that the two adjacent 3'-nucleotide identities are essential for editing at this position, but do not influence recognition of the first site located three nucleotides upstream.

3.2. Editing status of the cluster does not affect the activity of the RNA editing machinery

To investigate the influence of a nucleotide altered by RNA editing on the binding of the *trans*-factors of this cluster, a set of competition experiments was performed with competitors in various stages of editing.

3.2.1. Partially pre-edited, entirely unedited and fully pre-edited RNAs have comparable affinity to the RNA editing activity

To evaluate the respective binding affinities I tested the effects of incompletely pre-edited, of entirely unedited and of fully pre-edited RNAs as competitors. In a first set of experiments competition against a substrate pre-edited at the 5'-most site, site one, was analysed for editing at site three (Figure 3.5).

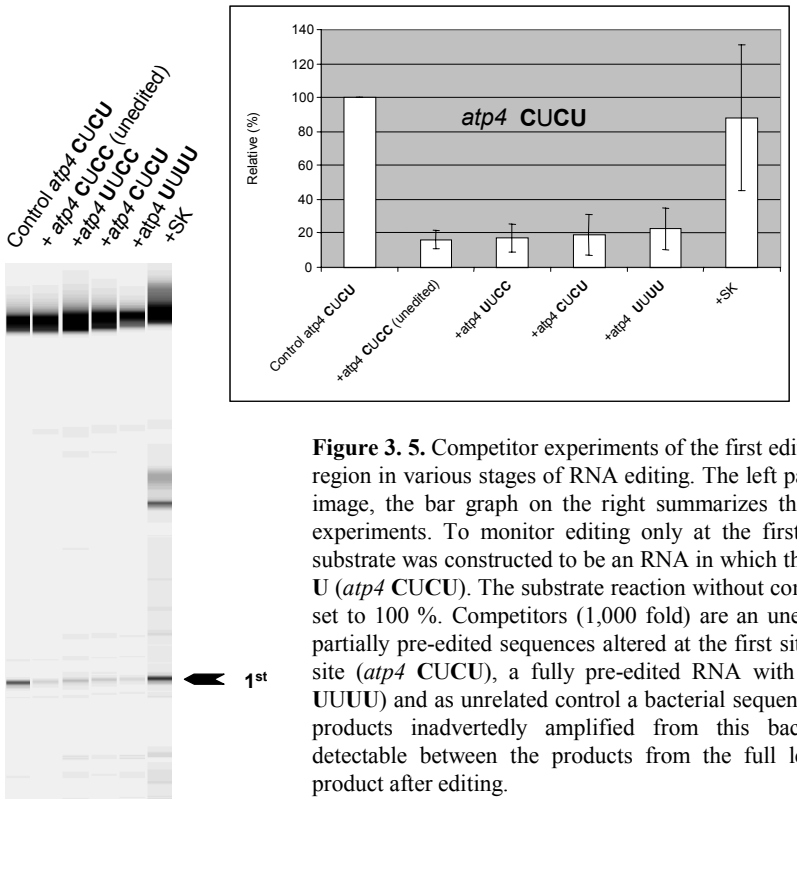


Figure 3. 5. Competitor experiments of the first editing site with the *atp4* substrate region in various stages of RNA editing. The left panel shows a representative gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the first site (arrowhead), the editing substrate was constructed to be an RNA in which the third site is already an edited **U** (*atp4* C**U**C**U**). The substrate reaction without competitor is run as reference and set to 100 %. Competitors (1,000 fold) are an unedited sequence (*atp4* C**U**C**C**), partially pre-edited sequences altered at the first site (*atp4* **U**U**CC**) or at the third site (*atp4* C**U**C**U**), a fully pre-edited RNA with all three sites changed (*atp4* **UUUU**) and as unrelated control a bacterial sequence (SK), respectively. RT-PCR products inadvertedly amplified from this bacterial competitor RNAs are detectable between the products from the full length substrate and the TDG product after editing.

All unedited, pre-edited and fully edited RNAs compete to comparable effect. In a second round of experiments a substrate pre-edited at site three, the 3'-most site, was assayed for the effect of the various competitors on editing at site one (Figure 3.6). Also at this site all fully and pre-edited competitors inhibit *in vitro* editing to an effect comparable to the non-edited

RNA. These results suggest that firstly the specificity mediating *trans*-factors for these RNA editing sites are present in limiting amounts, and secondly that these factors cannot discriminate between unedited and edited substrate RNAs.

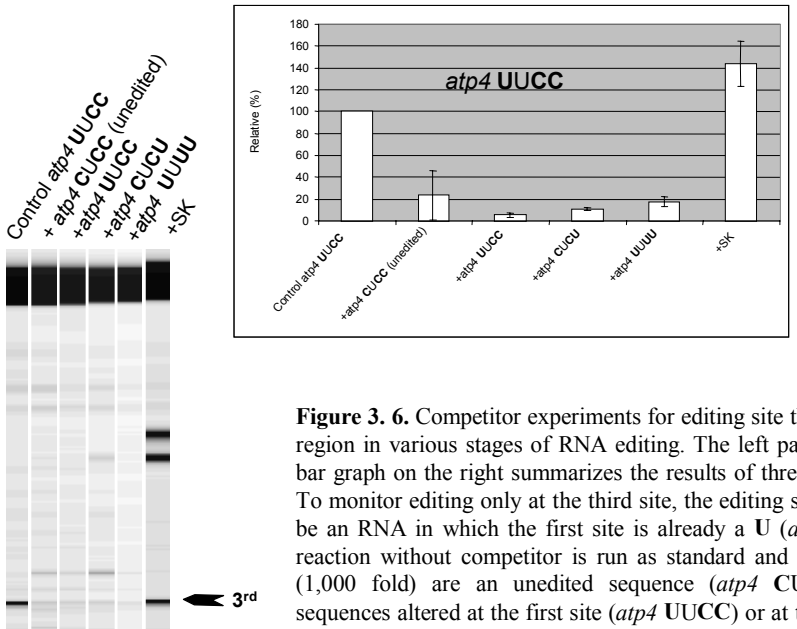


Figure 3. 6. Competitor experiments for editing site three with the *atp4* substrate region in various stages of RNA editing. The left panel shows a gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the third site, the editing substrate was constructed to be an RNA in which the first site is already a **U** (*atp4* **U**U**CC**). The substrate reaction without competitor is run as standard and set to 100 %. Competitors (1,000 fold) are an unedited sequence (*atp4* **C**U**CC**), partially pre-edited sequences altered at the first site (*atp4* **U**U**CC**) or at the third site (*atp4* **C**U**CU**), a fully pre-edited RNA with all three sites changed (**U**U**UU**) and as unrelated control a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full length substrate and from the TDG fragment after editing.

3.2.2. Binding of trans-*factors is independent of the editing site(s)*

To further analyze the influence of the editing status of a given site on recognition of this site by the *trans*-factors, I substituted the four nucleotides containing the three potential editing sites in the *atp4* template by continuous stretches of four A's and G's respectively and tested the effect of these RNAs as competitors (Fig. 3.7). Both sequences inhibited the *in vitro* reaction at a wild type template, suggesting that the limiting *trans*-factors bind upstream of the editing sites independently of the presence or absence of the edited nucleotide(s).

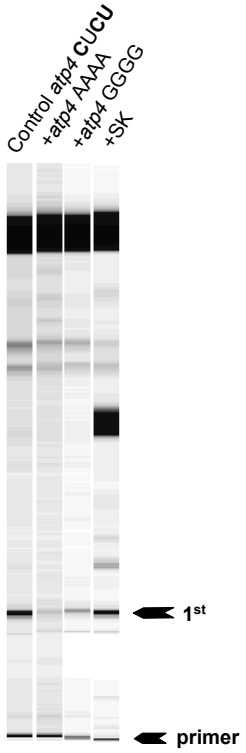


Figure 3.7. Competition with *atp4* RNAs in which the four pyrimidines covering the three editing sites are substituted by either of the purine. To monitor editing only at the first site, the editing substrate was an RNA in which the third site is preedited to a **U** (*atp4* **U**U**CC**). The substrate reaction without competitor is run as standard and set to 100 %. Competitors (1,000 fold) are either of the two purine sequences (*atp4* AAAA and *atp4* GGGG respectively) or as unrelated control a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full length substrate and from the TDG fragment after editing.

3.2.3. Specificity of the editing site recognition region

The nucleotide sequence of the specificity region for the three here investigated *atp4* editing sites is unique at least in the *Arabidopsis* mitochondrial genome and different from the sequence contexts at other editing sites. To investigate this specificity and the possibility that common *trans*-factors might recognize different sequences through some undetected common denominator in the vicinities of the various editing sites we tested the effect of this *atp4* specificity region on RNA editing at two different editing sites in the *atp9* mRNA (Figure 3.8). None of the fully, incompletely or un-edited *atp4* elements have any detrimental effect on editing at either of the two *atp9* sites *in vitro*. This result extends observations from several previous assays in mitochondria (and in plastids) which similarly suggested that indeed unique *trans*-factors recognize and bind to the various sequence elements around the about 30 plastid located editing sites and the more than 400 RNA editing sites in plant mitochondrial RNAs (Chaudhuri et al. 1995; Hirose and Sugiura 2001; Miyamoto et al. 2002 and 2004; van der Merwe et al. 2005).

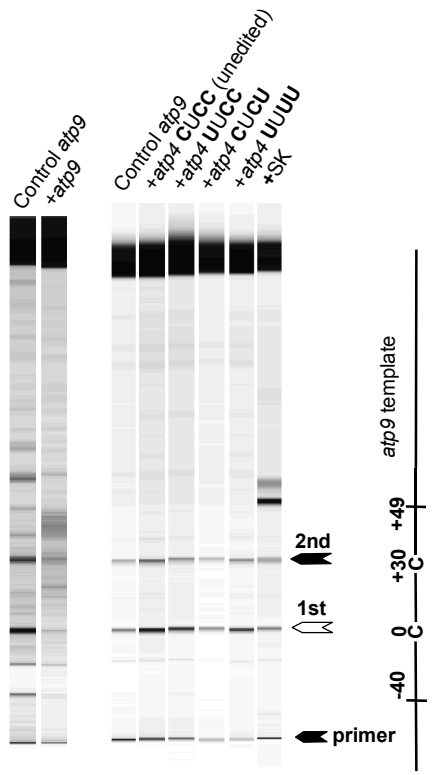


Figure 3. 8. Heterologous competitor experiments to test the specificity of trans-factor binding. Monitored were two *atp9* editing sites located 30 nucleotides apart for the effect of 1,000 fold competitors of the *atp4* substrate region in various stages of RNA editing. The locations of the two *atp9* RNA editing sites (1st and 2nd) are indicated in the schematic of substrate RNA (*atp9*) in the right hand margin. The substrate reaction without competitor is run as reference and control. Competition with the homologous RNA blocks RNA editing almost completely at each of the two sites, while heterologous RNA editing site sequences have little effect (Quantification of the gel data not shown). Heterologous competitors are an unedited *atp4* sequence (*atp4* **C**U**CC**), partially pre-edited sequences altered at the first site (*atp4* **UU**C**C**) or at the third site (*atp4* **C**U**C**U), a fully pre-edited RNA with all three sites changed (**UUUU**) and as unspecific and unrelated control a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full length substrate and the TDG product after editing.

3.3. Does the same recognition complex serve sites 1 and 3 from one or two cis*-elements?*

The above results extend several observations made on solitary RNA editing sites, e.g. the extent of the required 5´region needed for successful recognition is about 20 nucleotides in length for this cluster, similar to several singular mitochondrial and plastid editing sites (Chaudhuri and Maliga 1996; Farre et al. 2001). Site 3 of this cluster is successfully addressed only in the presence of two downstream nucleotides, suggesting that the size-specific modulation of the cluster lies in this 3`region and is not contained in the core recognition element. The sequence exchange at one editing site does not influence the recognition of another editing site in the cluster. The observations made in the reciprocal competition experiments with competitors of different editing status suggest that sites 1 and 3 of the editing cluster are addressed by the same recognition complex, but the actual process of recognition remains unclear. For example, it is still open if the recognition complex binds the *cis*-element at a single motif from where the enzymatic activity can reach both sites. Alternatively there may be two affinity motives within the essential *cis*-element serving sites 1 and 3 separately.

3.3.1. Alignment of the 20 nucleotides upstream region of sites 1 and 3 shows some shifted sequence similarity

Studies of several solitary editing sites suggest that these editing machinery does not tolerate any distance changes between the binding position of the recognition complex and the editing site (Neuwirt et al. 2005; Choury 2004). Comparison of the upstream region of editing sites 1, 2 and 3 of the cluster shows some sequence similarities at equal distance from sites 1 and 3 but no similarity for site 2 (Fig. 3.9). This observation suggests that within one *cis*-element two similar recognition motives maybe present to target respective sites 1 and 3 from equal distances. If true, the recognition complex would bind each of these two shifted *cis*-elements randomly. This can be concluded from the above detailed observation that the editing status of the cluster does not influence recognition, and that there is no order in the editing events at sites 1 and 3. It is possible, that site 2 of this cluster is addressed by an entirely different recognition complex which is not active in our *in vitro* system.

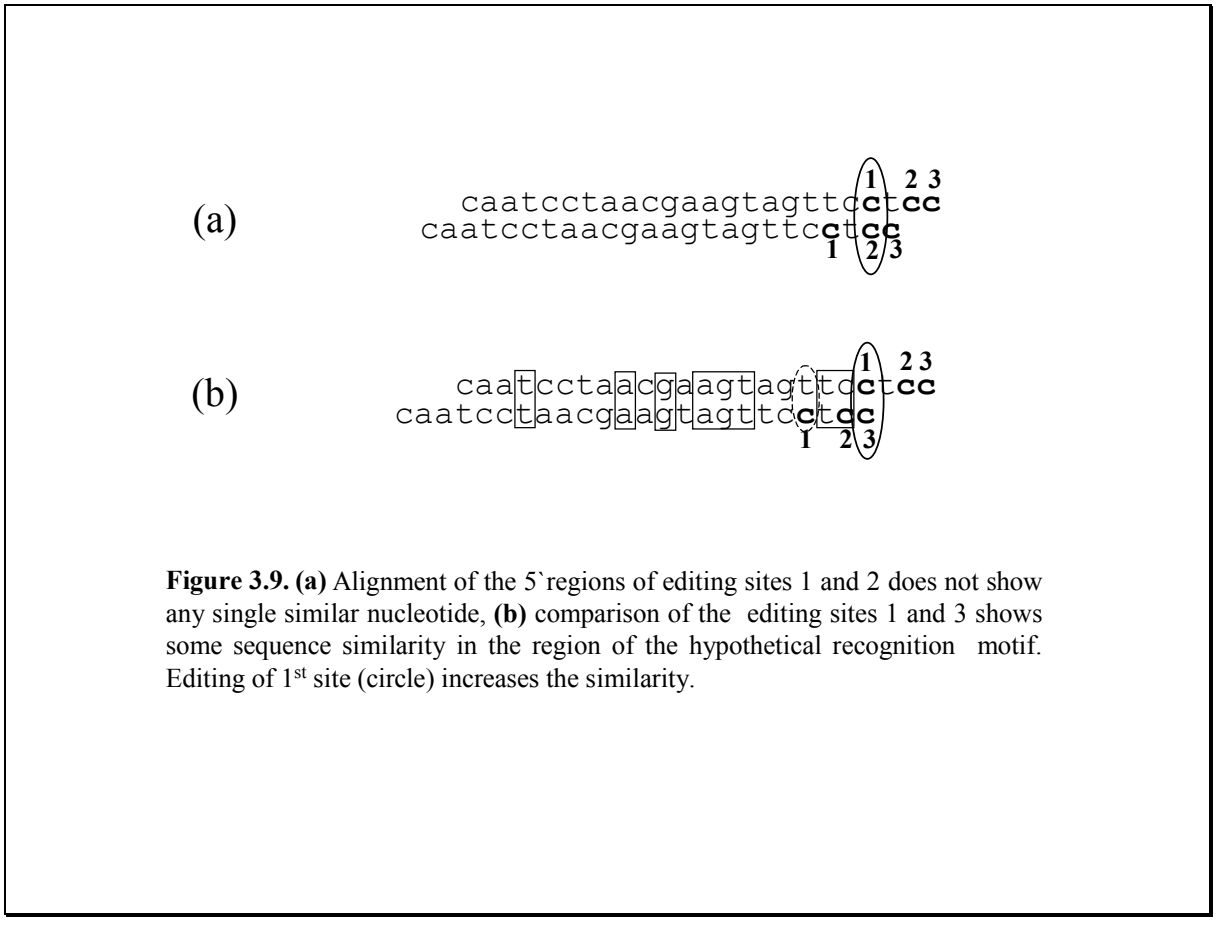


Figure 3.9. (a) Alignment of the 5`regions of editing sites 1 and 2 does not show any single similar nucleotide, **(b)** comparison of the editing sites 1 and 3 shows some sequence similarity in the region of the hypothetical recognition motif. Editing of 1st site (circle) increases the similarity.

If we assume that each editing event consists of three steps: assembly of the recognition complex, actual editing reaction and disassembly of the editing machinery, we would expect a

decreasing editing rate of consecutive sites such as the sites 1 and 3 in this cluster in such a model of overlapping recognition events, since the initiation of the recognition at site 1 would block access to site 3 for the editing machinery and vice versa. But, as detailed above, sites 1 and 3 of the cluster are edited with 100% efficiency *in vivo*. This indicates that only one recognition complex binds for sites 1 and 3. Moreover, as described above, *in vitro* most of the recognized molecules were edited at sites 1 and 3 of the cluster. However, of the RNAs edited only at one editing site, more were altered at the first editing site than at the third. This observation could suggest that the C to U change at site 1 introduces one more similar nucleotide into the hypothetical second recognition motif (Fig 3.9), and thus make these pre-edited RNAs slightly more attractive for editing at site 3.

3.3.2. Is the atp4 *cluster* cis*-element repeated at other editing sites in the mitochondrial transcriptome?*

Processing of the *atp4* editing cluster can perhaps be described as two independent recognition events on two individual homologous *cis*-elements which overlay each other and are shifted by 3 nucleotides. This speculation raises the question if there are any other editing sites in the mitochondrial transcriptome that are served by the same recognition complex.
In most editing sites analysed *in vitro* or *in vivo*, sequences within approximately 30 nucleotides (nt) 5' and 10 nt 3' of the edited C have been found to be required for selection of the correct editing target and for normal efficiency, but no global consensus sequences have been identified. Inspection of sequences surrounding chloroplast and mitochondrial editing sites reveals that some of them can be grouped with conserved nucleotides in the 30 nt 5' of the target C (Chateigner-Boutin and Hanson 2002). In the *atp4* gene the editing site at position S89L displays some nucleotide sequence similarity in the –21 area to the *atp4* editing cluster. This site also shows *in vitro* activity with a construct of –45+25 nucleotides around the editing position (Fig 3.19).

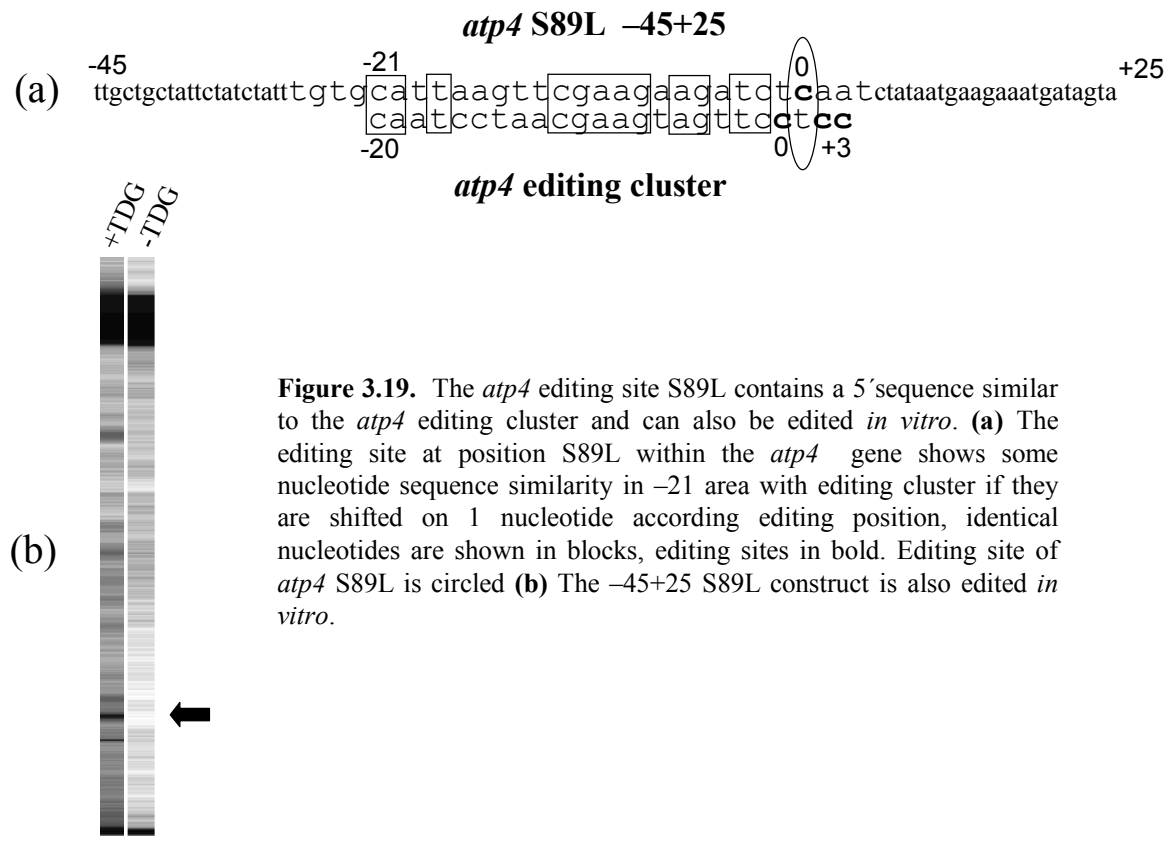


Figure 3.19. The *atp4* editing site S89L contains a 5´sequence similar to the *atp4* editing cluster and can also be edited *in vitro*. **(a)** The editing site at position S89L within the *atp4* gene shows some nucleotide sequence similarity in –21 area with editing cluster if they are shifted on 1 nucleotide according editing position, identical nucleotides are shown in blocks, editing sites in bold. Editing site of *atp4* S89L is circled **(b)** The –45+25 S89L construct is also edited *in vitro*.

To investigate if this site shares the recognition complex with the *atp4* editing cluster, a construct of -21+3 of the S89L *atp4* editing site and –20+5 of the editing cluster was cloned (Fig 3.20). It was expected that if these sites share the recognition complex they may show some reciprocal influence on the individual editing activity *in vitro*. The *in vitro* analysis of this template shows no editing activity at the editing site S89L but an enhanced type of the editing at the cluster editing sites 1 and 3 (Fig 3.20). However, the –20 +3 *atp4* S89L single and triple repeated are not active *in vitro*, possibly because the core recognition region for this site extends beyond –20+3. These data thus suggest that independent editing factors recognise these sites, but that the sequence similarity is sufficient to enhance editing at the *atp4* editing cluster. This effect suggests that the similar sequence of S89L editing site can be recognized by the *trans*-factors of the *atp4* editing cluster. Alignment of these sites (S89L and the cluster) shows that the sequence similarity is shifted by 1 nucleotide relative to the first editing positions. It is possible that specific *trans*-factor(s)/recognition complex can bind and/or can be assembled at the S89L *cis*-element but can not process this editing site due to the incorrect distance between binding site and C nucleotide**.**

To analyse if the intact functional *atp4* editing cluster recognition complex is able to assemble at the S89L editing site *cis*-element, a construct similar to the one described above but in which T at –1 of S89L editing site is substituted by C would have to be designed. *In vitro* editing of this construct at the new –1C of the S89L editing site would suggest that an editing recognition complex has the potential to serve different editing sites with sequence similarity. Another experimental assay to investigate this could be a heterologous competition of these two sites.

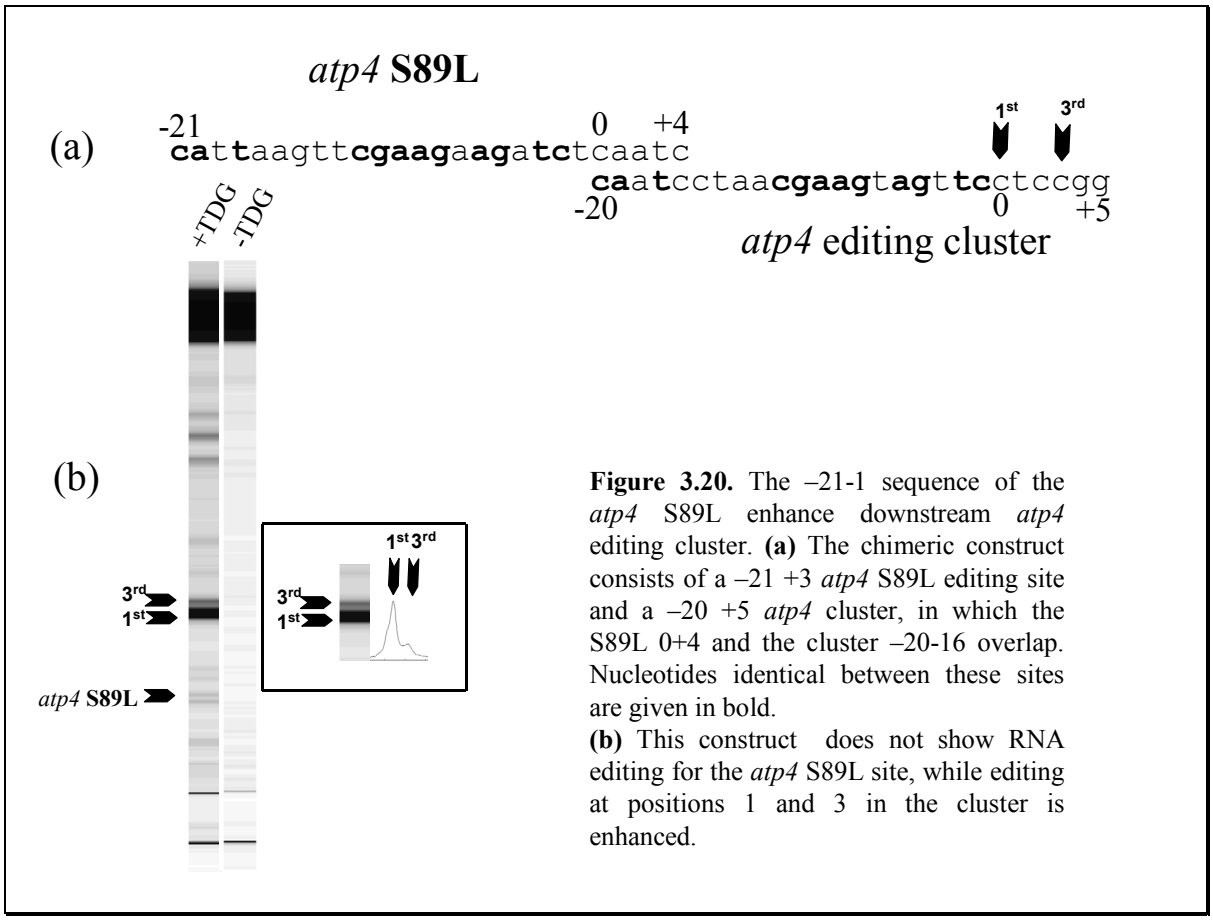


Figure 3.20. The –21-1 sequence of the *atp4* S89L enhance downstream *atp4* editing cluster. **(a)** The chimeric construct consists of a –21 +3 *atp4* S89L editing site and a –20 +5 *atp4* cluster, in which the S89L 0+4 and the cluster –20-16 overlap. Nucleotides identical between these sites are given in bold.
(b) This construct does not show RNA editing for the *atp4* S89L site, while editing at positions 1 and 3 in the cluster is enhanced.

3.4. Investigation of the RNA editing complex assembly

Previous research focussed on the recognition of isolated editing sites had concluded that recognition of these editing sites is most likely implemented by multisubunit complexes. Up to now the separate steps of the recognition and assembly process remain unclear: Is the assembly of the recognition complex initiated on the *cis*-element? What happens with the recognition complex after the editing reaction? Is it subsequently dissembled, or does it detach from the *cis*-element as an intact editosome that can serve further editing events?
Previously described competition experiments with heterologous competitors suggest that the specificity recognition factors are limiting compounds in the *in vitro* editing system. If the recognition of the editing site requires one (or more) specificity *trans*-factor present in limited amounts and assembly of the recognition complex is initiated at the *cis*-element, we can expect that a repeated *cis*-recognition motif in a given RNA molecule may be more attractive for assembly of the recognition complex and targeting of the editing site compared to single *cis*-elements. Such an element requires that the editing complex assembled at one *cis*-element can move in *cis* to other *cis*-element. If this linear movement is not possible the duplication should actually lower editing, because they could compete analogous to singular *cis*-elements present in *trans. Trans* as well as *cis* amplification of *cis*-recognition elements would deplete the limiting recognition factor from the assembly of an intact recognition complex.

3.4.1. Duplicated **cis-***element enhance* **in vitro** *editing*

To investigate the possibility that the *cis*-element is involved in initiating of the assembly of the RNA editing site recognition complex, we constructed a DNA clone for an RNA template in which the –20-1 region (without the edited C) is placed as a tandem repeat just upstream of the first editing site in the cluster. This construct allows to monitor editing site 1 of this cluster, without interference from editing site 3 which would need two more nucleotides downstream up to +5 to be edited as described above.
In vitro editing of this construct yields about 50% of editing whereas a single –20+3 shows average editing of about 2% (Fig 3.10).

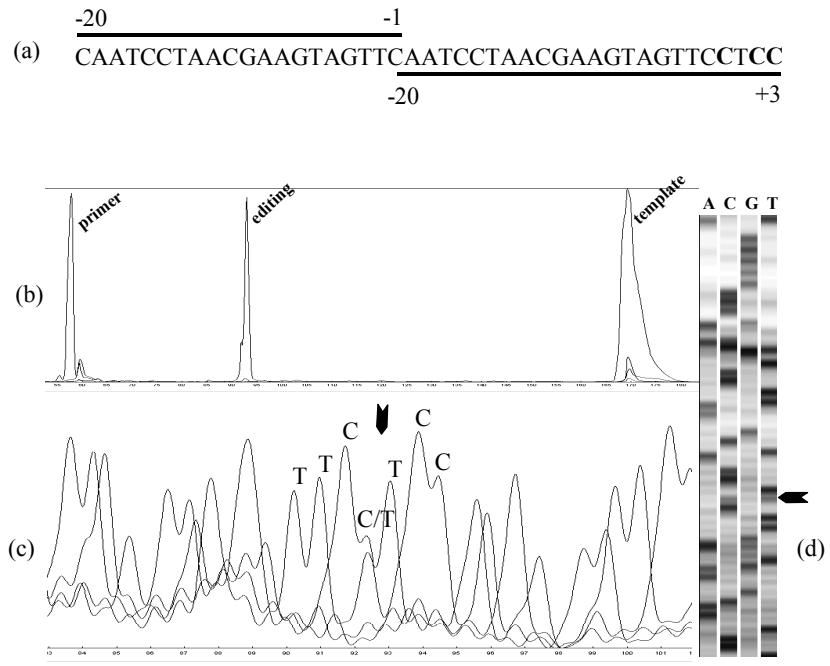


Figure 3.10. -20-1 sequence of *atp4* enhances the editing activity. *In vitro* analysis of the *atp4* –20+3 RNA template with an upstream duplication of the –20-1 nucleotides **(a)** shows an editing efficiency of about 50% **(b).** The sequencing gel of the *in vitro* PCR product **(c)** and the chromatogram **(d)** give a double reading of C and T at the first editing site.

3.4.2. Four times repeated –20+3 constructs are edited in vitro *50% to 90%*

As described above, a duplicated *cis*-element enhances the editing activity *in vitro*. However, we can not exclude the possibility that the enhancing effect is caused by the artificial structural property of this template. To verify the enhancing effect of the repeated *cis*-elements a four times –20+3 repeated template was constructed. In this template the bacterial vector sequence recover –23 nucleotides at the first repeat and +5 nucleotides at the terminal repeat. As previously noted the single –40+20 construct shows higher affinity for the editing complex than the –20+20 construct. Moreover, the +5 vicinity at the fourth repeat allows to monitor sites 1 and 3 of the terminal cluster. *In vitro* analysis of this constructs shows from 50% up to 90% of the editing activity (Fig 3.11).

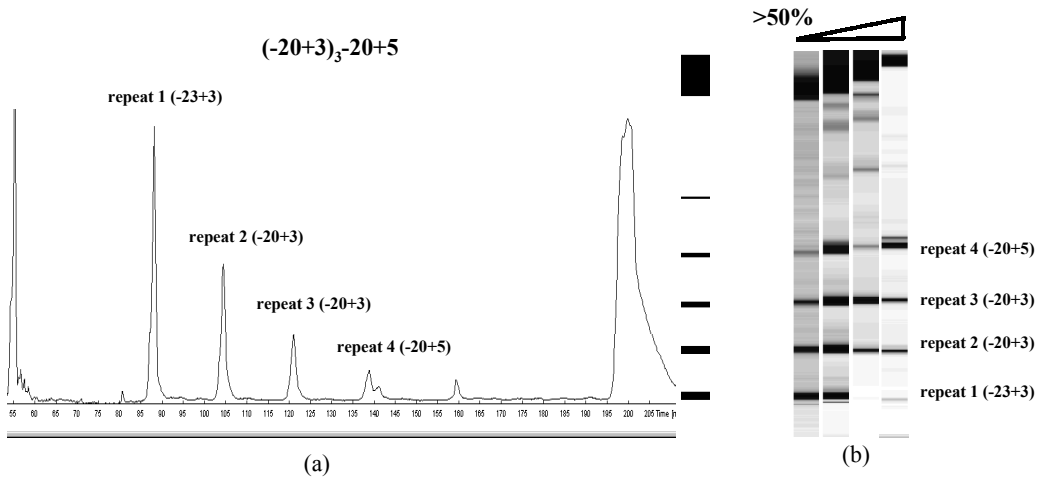


Figure 3.11. *In vitro* analysis of the *atp4* RNA template which contains the –20+3 region repeated four times (in the first repeat the bacterial sequence recovers -23 nucleotides, in the last repeat +5) **(a).** If the editing efficiency is around 50% and several editing events occur randomly on different or on the same RNA molecule, the TDG treatment will give numerous DNA fragments, with only the fragment digested at the first repeat editing site carrying the Cy5 label and all other fragments derived from this molecule being invisible on the gel. Consequently each signal on the gel and chromatogram is diminished by 50% from the previous**. (b)** When from 60 up to 90% of the RNA molecules have been edited, the majority of the T/G mismatches will correspond to the least edited repeat. This will lead to domination of the signal at repeats 2, 3 and 4.

If the editing efficiency is around 50% and individual editing events occur randomly distributed between the different RNA molecules, the TDG treatment of the *in vitro* RT-PCR product will give several DNA fragments from editing events in the same RNA molecule. However, only the fragments digested at the editing site of the first repeat carry the Cy5 label. Since the efficiency of the TDG enzyme is around 60-80%, all other fragments derived from the given molecule show less intensive signals on the gel and chromatogram. In the case when from 60 to 90% of the RNA molecules have been edited, the majority of the T/G mismatches after the reannealing reaction will correspond to the least edited repeat. This will lead to domination of the signal at repeats 2, 3 and 4 (Fig 3.11b).

The sequence of 19 individual clones derived from the RT-PCR product confirm an *in vitro* RNA editing reaction efficiency of about 50% in this particular experiment (Fig 3.12). The profile of the editing events among individual clones shows that most of the successfully recognised molecules have been edited at the first repeat, with much fewer molecules also edited at the fourth repeat. The frequencies of editing events at repeats 2 and 3 are identical. At present one cannot exclude that the editing activity at the first repeat is higher because it

has a –23 upstream vicinity rather than –20 in the other repeats and therefore may be more attractive for the *trans*-recognition elements than the three other repeats.

Clone	1st rep.	2nd rep.	3rd rep.	4th rep.
1	+	+	+	+/+
2	+	+	+	+/-
3	+	+	+	+/-
4	+	+	+	-/-
5	+	+	+	-/-
6	+	+	+	-/-
7	+	+	+	-/-
8	-	+	+	+/-
9	+	-	-	-
10	+	-	-	-
11-19	-	-	-	-

Figure 3.12. Sequence analysis of 19 individual clones obtained from the RT- PCR product of the *atp4* $(-20+3)_3$-20+5 construct shows an *in vitro* editing efficiency about 50% (successfully edited positions are given as **+,** at the 4th repeat ***/*** indicates editing at sites 1 and 3). Interestingly, when the editing site at the first repeat has been successfully edited, downstream repeats are edited without interruptions (up to the 4th) .

The observation that editing sites at repeats 2 and 3 are more frequently edited than the editing sites in the terminal repeat can be explained by that the second and third repeats are surrounded by identical editing sites in upstream and downstream, while repeat 4 has such a repeat only on the upstream side.

3.4.3. The enhancing editing effect of the repeated **cis***-elements is size specific*

To further investigate the origin and nature of the enhancing effect a construct with a six times repeated –20+20 region was cloned. *In vitro* processing of this template yields an average of 2% editing efficiency at sites 1 and 3 of each repeat. From this we can conclude that the distance between editing sites in repeated constructs is crucial for the generation of

the enhancing effect. One possible explanation for this observation is that the minimal size sufficient for recognition repeated *cis*-elements attract more recognition elements in *trans.* And recruited in a range about of 20 nucleotides recognition factors may be have a better chance to interact with each other to rebuild a functional multisubunit recognition complex, than broken up during the mitochondrial lysate preparation. But if the distance between editing sites in such repeated constructs increases only on 17 nucleotides as in $(-20+20)_6$ *atp4* RNA the enhancing effect is disappearing. It can mean, that specificity *trans*-factors have less probability to interact with each other and to be assembled in the functional recognition complex.

3.4.4. Editing events at the cis*-element duplication and in a downstream –40+20 fragment of the editing cluster are connected*

To investigate the possibility that one particular recognition complex can serve more than one recognition event we constructed a clone for an RNA template that consists of the enhancing element described above (-20-1-20+3) with a –40+20 fragment of the *atp4* editing cluster at a distance of 18 nucleotide downstream. This construct contains three repeats of the *atp4* cluster with the third separated by 60 nucleotides from upstream two. This construct should allow to determine if the enhancing effect is generated only at the -20-1-20+3 duplication or also at the –40+20 fragment 60 nt distant. Thus this construct permits to monitor a potential physical *cis* connection between distant editing events. The *in vitro* editing of this construct yields about 40% editing at the *cis*-element duplication (Fig 3.13) and slightly increased editing activity of sites 1 and 3 in the –40+20 fragment.

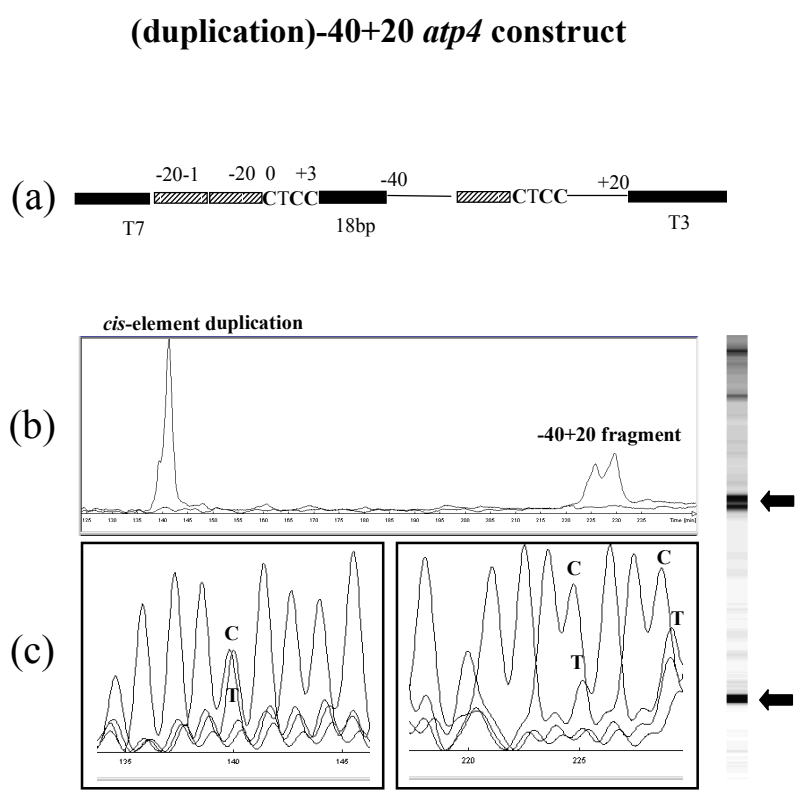


Figure 3.13. The *cis*-element duplication enhances editing at –40+20 fragment separated by 60 bp. **(a)** Cartoon of the construct with the *cis*-element duplication and the –40+20 *atp4* fragment (black bars show the vector parts). **(b)** *In vitro* analysis of the (duplication)-40+20 construct. **(c)** Direct sequence of the *in vitro* RT-PCR (duplication)-40+20 *atp4* product shows about of 50% editing at the duplication editing site (left) and about 15% of editing at the –40+20 fragment (right).

Sequence analysis of individual clones derived from the *in vitro* RT-PCR product shows that editing at the –40+20 fragment occurs only on RNA molecules that have been processed at the *cis*-element duplication. From 30 sequenced clones 12 are edited at the *cis*-element duplication, equivalent to 40% editing at this position. Out of these 12 clones one was edited at site 1 of the cluster at –40+20 fragment and another at sites 1 and 3. No editing events were found in the –40+20 fragment in clones unprocessed at the *cis*-element duplication. For further evaluation of the connection between distal editing events, an RT-PCR with a downstream primer complementary to edited –40+20 fragment molecules was performed. Direct sequencing of this RT-PCR product shows a significant increase of the T signal in the sequencing gel and chromatogram at the duplication editing position (Fig 3.14). This result suggests that most of the editing events in the –40+20 fragment occur at RNA molecules also recognised at the *cis*-element duplication. This observation suggest that editing events at the *cis*-element duplication and –40+20 are connected.

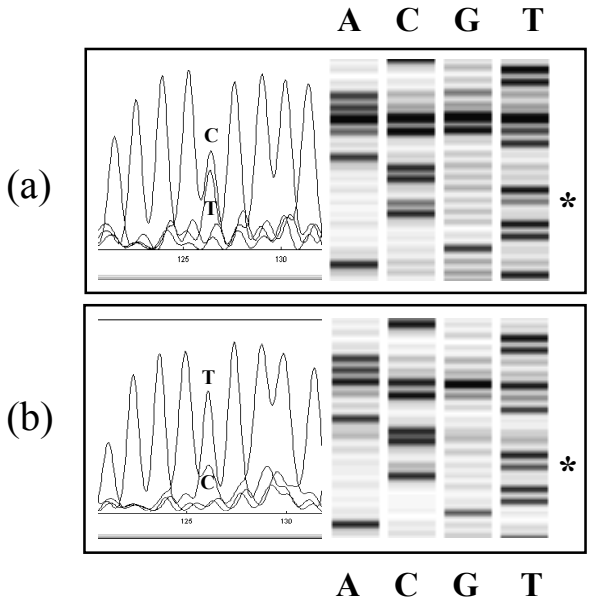


Figure 3.14. Editing events at the cis-element duplication and the –40+20 fragment are connected**. (a)** Direct sequence of the *in vitro* RT-PCR product of the (duplication)-40+20 construct shows C/T double reading position (*) at the duplication editing site, that displays about 40% of editing. **(b)** Direct sequence of the selective (only molecules edited at –40+20 fragment are amplified) RT-PCR product of the (duplication)-40+20 construct shows a significant increase of the T signal at this position.

A possible explanation of this effect is that the recognition complex assembled at the duplication proceeds along a given RNA molecule to process the downstream editing cluster of the –40+20 fragment. To investigate this possibility of a linear scanning of the RNA editing complex, a construct was designed which contains a –179+20 fragment of the *atp4* gene 18 nucleotide downstream of the *cis*-element duplication. This construct allows to monitor if there is a connection of editing events when the *cis*-element duplication and this editing cluster are separated by 200 nucleotides. If there is a linear progressive screening mechanism of editing site recognition along the entire molecule the distance between *cis*-element duplication and downstream editing cluster should not influence the distal editing events.

3.4.5. Editing events at the cis*-element duplication and placed downstream –179+20 fragment of the editing cluster are not connected*

In vitro analysis of this construct (duplication)-179+20 shows editing at both the *cis*-element duplication and at the –179+20 fragment. The editing efficiency at these positions are higher

than reported for a single *atp4* editing cluster. To analyse the connection of these editing events a selective RT-PCR with a downstream primer complementary to the RNA molecules edited at the –179 +20 fragment was performed. (Fig 3.15)

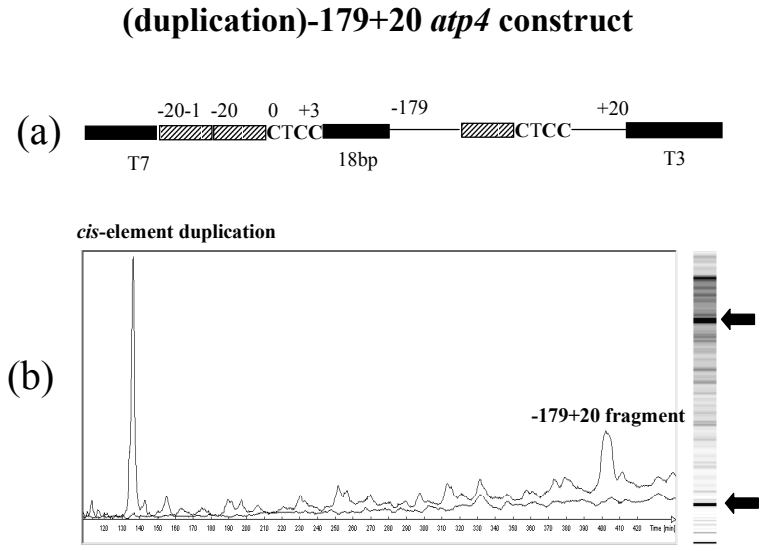


Figure 3.15. The *cis*-element duplication does not enhance editing at the –179+20 fragment. **(a)** Cartoon of the construct of the *cis*-element duplication and the –179+20 *atp4* fragment (black bars indicate vector parts of the construct). **(b)** *In vitro* analysis of the (duplication)-40+20 construct shows enhanced editing at the duplication editing site and increased editing at the –179+20 fragment.

Direct sequencing of this RT-PCR product does not shows a significant increase of the T signal in the sequencing gel and chromatogram at the duplication editing position (Fig 3.16). This indicates that there is no connection in the editing events along this particular RNA molecule. Thus, a connection between editing events spaced along a particular RNA molecule is distance dependent. This observation suggests that there is no a linear progressive screening mechanism of editing site recognition along an entire RNA molecule but the such a scanning only occurs over distances of 1 ≈50 nucleotides where after the editing complex dissociates from the RNA or disassembles.

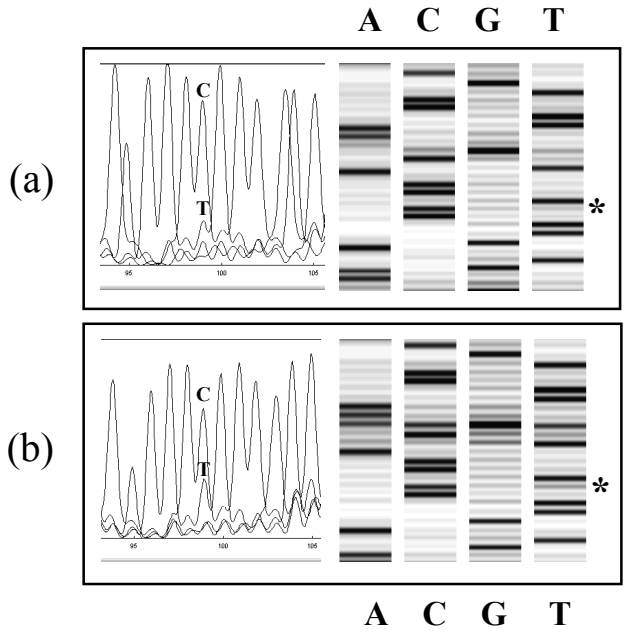


Figure 3.16. Editing events at the *cis*-element duplication and the –179+20 fragment are not connected**. (a)** Direct sequence of the *in vitro* RT-PCR product of the (duplication)-179+20 construct shows the majority of C in the double reading position (*) at the duplication editing site. **(b)** Direct sequence of the selective (only molecules edited at –179+20 fragment are amplified) RT-PCR product of the (duplication)-179+20 construct shows slight increase of the T signal at this position.

3.5. Is the in vitro *enhancing effect of the repeated* cis*-element also typical for singular editing sites?*

As described above repeated *cis*-elements strongly enhance the *in vitro* editing activity of the *atp4* editing cluster. To investigate if this effect can also be at singular editing sites, several constructs with various repeats of the first editing site of the *atp9* gene were cloned. The –20+5 *atp9* construct is not edit in the cauliflower *in vitro* system. To determine the minimal sequence sufficient for recognition of the first *atp9* editing site a –25+3 construct was cloned. This construct is successfully recognised *in vitro* with an average efficiency of 2% (Fig 3.17b). Surprisingly, an *in vitro* experiment with two times repeated $(–20+5)_2$ *cis*-element shows an induction of the editing activity at the first repeat (Fig 3.17).

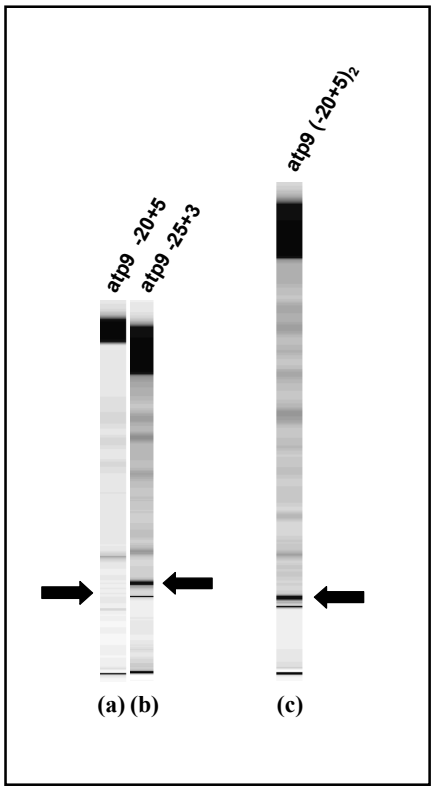
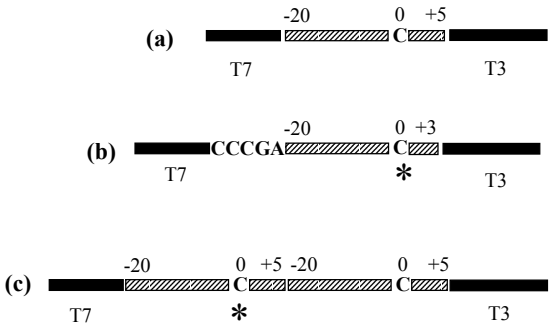


Figure 3.17. Duplication of the –20+5 *atp9* fragment induces editing activity. **(a)** The –20+5 fragment of *atp9* gene shows no activity *in vitro.* **(b)** The –25+3 fragment of *atp9* gene is edited *in vitro*. **(c)** The construct with a duplicated $(–20+5)_2$ *atp9* fragment shows editing of the upstream editing site.



However, the editing activity for this construct is not enhanced but displays the average value also observed with the –25+3 template RNA. To further investigate the enhancing effect of singular editing sites a construct containing of three tandemly repeated *atp9* fragments was cloned. The first repeat of this construct is –25+3, the following two are –22+3 each. This construct shows an enhanced editing *in vitro* (Fig3.18a).

As an additional negative control experiment a construct in which three repeats were mutated by complimentary substitution in the –9-5 region was cloned. This construct has no *in vitro* activity (Fig 3.18b).

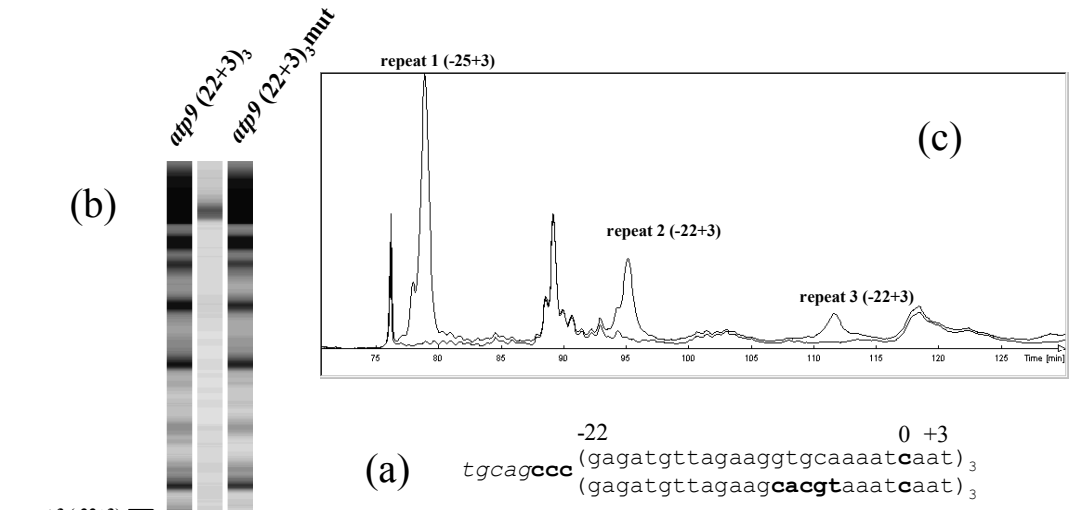


Figure 3.18. Tandem repeats of the cis-element enhance the editing activity of *atp9*. **(a)** Diagram of the $(-22+3)_3$atp9 and $(-22+3)_3$atp9 mutated constructs. In the first repeat the bacterial sequence recovers -25 nucleotides. Mutated nucleotides are given in bold. **(b)**The *in vitro* analysis of the tandem repeated *cis*- elements (-22+3) of the *atp9* gene shows an enhanced level of editing (left lane). The template with mutated repeats (in –9-5) shows no activity *in vitro* (right lane). **(c)** The patterns of the TDG products in chromatogram suggest that most of the recognized RNAs are edited at all positions.

4. Discussion

Previous studies of the RNA editing in plant mitochondria focused on the investigation of the singular editing sites. Here I investigate the *in vitro* processing of the *atp4* editing cluster. Analysis of the physically connected editing sites gives an opportunity to study a different aspects of the RNA editing, such as a *cis*-element extension for recognition of editing sites, behaviour of the recognition complex, assembly of the editing machinery etc. Moreover, investigation of such an editing system helps to gain an information about complicity of numerous editing sites in the complicated editing process in plant mitochondria.

4.1. Editing sites 1 and 3 of the cluster are altered by the same editing machinery, editing site 2 most likely requires a distinct recognition complex

The observation that the editing sites 1 and 3 of the *atp4* editing cluster show correlated *in vitro* editing activity using a different lysate preparations suggests the similar editing machinery for these sites. Moreover, analysis of the *in vitro* editing profile shows that most of the RNAs have been edited at sites 1 and 3 with several examples of independent events at sites 1 and 3 also being found, in which 1 is recognised more frequently. These observations suggest that there is no consecutive order in the recognition of sites 1 and 3 in this cluster and that these sites are served randomly.

The *in vitro* analysis of templates with different distributions of the upstream and downstream regions of the cluster shows analogous requirements of the upstream *cis*-elements for sites 1 and 3. Some recognition modulation of site 3 is noted downstream, where it needs two downstream nucleotides to be edited. This can be explained by the recognition complex requiring some kind of support in 3´to make the C to be edited accessible for the editing enzyme. Another possible explanation of this effect could be an unspecific influence of the downstream vicinity of the bacterial vector on the editing efficiency of site 3. The competition experiments with competitors in different editing states also support analogous requirements of the *cis*-elements for the sites 1 and 3, since the different RNA substrates compete the editing activities at the sites 1 and 3 with equal efficiency.

Since editing of the site 2 of the cluster was never observed in *in vitro* experiments with constructs of different editing status/different *cis*-element extensions and using enhancing templates, I suggest that site 2 of this cluster needs some additional (hypothetical) cofactor that is not present/abundant in the *in vitro* system. Also, the delayed editing rate of site 2 *in*

vivo suggest distinct possibly less abundant recognition complex serves this site. Delayed editing of site 2 would be of no function consequence since this is a silent event once site 3 is edited.

4.2. The recognition complex for sites 1 and 3 binds the **cis**-*element at two affinity motifs shifted by 3 nucleotides*

Assuming that editing sites 1 and 3 are served by the same editing complex two scenarios of action of the editing complex are possible: in the first, the recognition complex binds the RNA at one particular region and serves both sites from one position. According to this scenario the recognition complex and/or the editing enzyme will have to be able to stretch from this position to reach sites 1 and 3 which are separated by 3 nucleotides. But in the *in vitro* experiments I never observed editing at the –1 position which is also a C or at the second editing site. In other words, if the editing complex stretches between editing sites 1 and 3, it is unclear why it does not act at the –1 and +2 cytidines. Furthermore, at other singular mitocondrial and plastid editing sites the respective editing complex does not tolerate single nucleotide distance changes between the binding position and the nucleotide to be edited (Choury et al. 2004; Neuwirt et al. 2005).
In the second scenario the recognition complex binds two affinity overlapping motifs present in one *cis*-element and shifted by a few nucleotides. Alignment of the upstream region respective to sites 1 and 3 shows some sequence similarity at equal distances, but no similarity at the respective distance to site 2. These observations suggest that the recognition complex binds the *cis*-element at two positions for sites 1 and 3 with equal distances between the binding positions and the sites.

4.3. Recognition of the editing cluster behaves as recognition of 3 singular editing sites

The above observations allow to describe editing of the *atp4* cluster as three independent recognition events. The overlapping binding sites can lead to competition between the individual recognition events, which might influence the individual editing rates *in vivo*. While editing sites 1and 3 are most likely served by the same recognition complex that binds the *cis*-element and then within this element rapidly shifts to the overlapping element editing

site 2 should be served by a distinct recognition complex. The one recognition complex serving sites 1 and 3 will with higher probability serve these two editing sites rather than being released to make the *cis*-element accessible for site 2. Consequently, *in vivo* 100% editing of sites 1 and 3 and a somewhat reduced editing rate for site 2 are observed.

4.4. Specificity determinants do not include the nucleotide(s) to be edited

The observation that the editing activity binds to unedited as well as edited RNA molecules suggests that the *trans*-acting specificity factors do not recognize the nucleotide to be edited. In the special situation of these three clustered editing sites in the *atp4* mRNA, a single upstream *cis*-region acts as anchor for editing sites 1 and 3. *In vitro* these sites are correctly addressed, since editing of both sites is lost upon deletion of nucleotides closer than 20 nucleotides upstream (data not shown). The editing complex does recognize the identity of the nucleotides downstream of the last editing site for contact at this site, but must also keep the C to be edited, accessible for contact by the actual editing enzyme(s). This latter conclusion is confirmed by the efficient competition of RNA molecules in which the four pyrimidines covering the three editing sites are substituted by either purine. These experiments suggest that the identity of the to-be-edited nucleotide has no influence upon recognition of the specific editing site by the trans-acting factor(s).

Two scenarios can alternatively explain these observations: In the first, the editing enzyme itself is not sequence-specific and is guided to an editing site by the separate specificity factor(s). These latter do not contact the nucleotide to be edited, but allow the enzyme to approach the RNA and to perform the editing reaction. The sequence-specific binding proteins can additionally contact the downstream nucleotide(s), but must leave a gap for the enzyme approach. On the other hand the editing enzyme itself could have some steric preferences which require certain nucleotide identities in this downstream region. However, considering the large number of editing sites in plant mitochondria with very different downstream nucleotides, this latter constraint appears to be less likely. In the alternative scenario, instead of an assembly of several proteins with these distinct (partial) functions, a single *trans*-acting protein may contain a respective enzymatic C to U deamination domain and there may not be a separate editing enzyme. This single protein would have to recognize the specificity region, bind to the RNA without regard to the editing status of this or other editing sites and attempt to alter the target nucleotide.

4.5. Incompletely edited RNAs can be substrates for RNA editing

The observation of incompletely as well as fully edited mRNAs in the steady state RNA population of plant mitochondria about 15 years ago has immediately raised the question of whether these incompletely edited RNAs are terminally abandoned errors or whether they represent intermediates, in which RNA editing can still be completed (Hiesel et al., 1990; Schuster et al., 1990). This question has not been decided to date, since there was no experimental assay available to follow the fate of such incompletely edited molecules. The development of reliable *in vitro* RNA editing systems has now allowed to address this long-standing debate and to provide a clear answer.

In the cauliflower *atp4* mRNA the constellation of three closely spaced sites offers a unique opportunity to investigate the processivity of the RNA editing complex in plant mitochondria and to test whether its binding affinities are influenced by the editing status of the RNA substrate. In the direct approach incompletely edited substrates show comparable *in vitro* RNA editing at the respective unedited sites. The indirect assay, in which incompletely and fully pre-edited RNAs are employed as competitors, corroborates this result, since these molecules compete as effectively as the completely unedited RNA.

These experiments now show that incompletely edited RNA molecules can clearly act as competent substrates and thus have to be considered intermediates in the processing step of RNA editing. This result furthermore makes it unlikely that these intermediates could give rise to a group of slightly variant proteins with modified properties in plant mitochondria (Grohmann et al, 1994; Lu and Hanson, 1994; Phraener et al, 1996). The more probable scenario is that functional proteins will only be specified by fully edited RNAs as supported by protein sequence analysis (Grohmann et al, 1994; Lu and Hanson, 1994).

4.6. Assembly of an editing site recognition complex

Investigation of the first editing site in the atp9 transcript editing with the mitochondria *in vitro* system suggests that addressing is possibly performed by polysubunit machinery (Takenaka et al. 2004). For the human apoB mRNA editing, specific protein-RNA interactions 3´of the editing site are necessary for site-specific editing and for assembly of the multisubunit editosom (Mehta and Driscoll 2002).

4.6.1. **Cis-***elements can be involved in recognition complex assembly*

In vitro experiments with polymeric constructs show that two or more *cis*-elements located in tandem along an RNA template can induces enhancing effect. This enhancing editing effect is distance sensitive and is effective if the editing positions are located about 25 nucleotides distant, but disappears if this distance is 40 nucleotides.
A possible explanation of this behaviour of the enhancing effect is that all/most of the recognition factors of a given recognition complex have strong affinity to a nucleotide sequence of a given *cis*-element which they need to assemble into a complex with the editing enzyme. Two or more *cis*-elements then attract possibly more recognition factors. Once assembled the editing complex can move to nearby identical editing site fully intact or, alternatively, was rapidly reassembled at next identical editing site.

4.6.2. The same **trans-***factors/recognition complex can serve several editing events*

As described above, it is likely that the recognition complex assembly is initiated at the *cis*-element. But the fate of the recognition complex after editing reaction is still unclear. There are two possible scenarios. In the first, the recognition complex releases the RNA substrate by dissociation and disassembly of the complex. In the second, the recognition complex detaches the *cis*-element as an intact editosome which can rapidly serve another round at nearby site.
The observation that the (duplication)-40+20 *atp4* construct shows a connection between editing events at the *cis*-element (duplication) and at the –40+20 fragment, suggests that the same recognition complex can serve more than one round of editing. Thus this observation most likely suggests that the recognition complex move linear in *cis* fore some distance. Here I can not decide whether the assembled complex shifts or if the complex dissembles and the probability for re-assembling is increased at a nearby recognition motif.

4.6.3. Editing sites are more likely recognized by a touch-and-go mechanism rather than by linear progressive screening

Up to now the processivity of the RNA editing recognition remains unclear. Two alternatives are possible, in the first the recognition complex screens for a specific editing site along the

entire RNA molecule, in the second it binds the specific editing site in a touch-and-go manner The *in vitro* experiment with a four times repeated *cis*-element shows more frequent editing at the first repeat. This observation can be explained by that the first repeat of this construct has a longer upstream (23 nucleotides) region of the *cis*-element than repeats 2, 3 and 4 (20 nucleotides) and the recognition complex can bind the first repeat more efficiently. The editing events at the second and third repeats are equal by frequent and double the editing frequency at the fourth repeat. If the recognition complex is hopping from one editing site to another along the RNA molecule, repeats 2 and 3 have twice the chance to be recognised because they are surrounded by other repeats in contrast to repeat 4 with only one upstream neighbour. From this reasoning there would be no preference of directing for the association of the editing complex as a touch-and-go movement along the RNA.

Supporting this model in the *in vitro* analysis of the (duplication)-40+20 and (duplication)-179+20 *atp4* constructs, which show a clear distance dependence between the editing events within an RNA molecule.

These observations thus suggests that two homologous *cis*-elements located closer to each other give the respective editing complex the possibility to hop from one editing position to the next and process both of them with increased probability. RNA editing sites in plant mitochondria are thus most likely recognised in a touch-and-go manner.

4.7. Editing sites with similar surrounding sequences can be recognized by the same **trans-** *factors*

In most editing sites analysed *in vitro* or *in vivo*, sequences within approximately 30 nucleotides (nt) 5' and 10 nt 3' of the edited C have been found to be required for selection of the correct C editing target and for efficient editing, while no general consensus sequences have been identified. Inspection of sequences surrounding chloroplast and mitochondrial editing sites reveals that they can be sorted into groups of conserved nucleotides within 30 nt 5' of the C target of editing (Chateigner-Boutin and Hanson 2002). In addition the finding that non-cognate mitochondria are able to edit residues that are not edited in their own transcripts (Choury and Araya A. 2006) support the hypothesis that the same *trans*-acting factors may recognize several editing sites.

The *in vitro* analysis of the heterologous construct consisting editing sites S89L and the cluster of the *atp4* gene shows enhanced editing at sites 1 and 3 of the *atp4* cluster but no

editing at the S89L editing site. This result suggests that the S89L editing site *cis*-element attracts the *trans*-factor(s) also serving the editing cluster and enhances editing of the cluster. However, the recognition complex of the editing cluster is not able to identify the target C of this S89L editing site within this particular construct. It is possible that the region of the S89L *cis*-element present in this construct is not sufficient for successful addressing of this site. The most likely explanation for this observation is that the target C of S89L site is shifted by 1 nucleotide downstream of the conserved recognition motif in the respect to the cluster, which the recognition complex can not tolerate.

5. Materials and Methods

5.1. Preparation of mitochondrial extracts

Mitochondria of cauliflower inflorescences were isolated by differential centrifugation and purification on Percoll gradients as described previously (Binder et al., 1995). Four hundred mg of purified mitochondria were lysed in 1,200 µl of extraction buffer (0.3 M HEPES-KOH pH 7.7, 3 mM magnesium-acetate, 2 M KCl, and 2 mM dithiothreitol) containing 0.2% Triton X-100. After 30 min of incubation on ice, the lysate was centrifuged at 22,000 x g for 20 min. The supernatant was recovered and dialyzed against 5 x 100 ml dialysis buffer (30 mM HEPES-KOH pH 7.7, 3 mM magnesium-acetate, 45 mM potassium-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. When stored at -80 °C the lysate was stable for at least 3 months.

5.2. RNA substrates

5.2.1. Cloning procedure

Standard cloning was done using the *E. coli* laboratory strain DH5α and the vector pBluescript SKII$^+$ or the modified pBluescript SKII+ for the *in vitro* substrates according to the description in Takenaka and Brennicke (2003). The protocol for cloning of the repeated constructs was modified. To create direct repeats of the editing site fragments, PCR products treated according to standard procedure were pre-ligated for 4h, then the bacterial vector was added to the ligation reaction. *Escherichia coli* was transformed by electroporation using the Gene Pulser from BioRad according to the recommendation of the provider. The selection of the positive colonies was performed by PCR with T7 and downstream insert specific primers. The constructs for competitors were cloned into the vector without the KS sequence to prevent co-amplification of the competitor substrate.

5.2.2. In vitro *transcription*

DNA clones were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the template RNA. The synthesized RNA molecule contains a fragment of the cauliflower mitochondrial gene flanked by bacterial sequences to allow specific amplification from these bacterial sequences against the background of endogenous mRNAs. Different parts of the *atp4* and *atp9* genes were inserted into the vector in EcoRV or SmaI restriction endonuclease sites of the multiple cloning site between the T7 promoter upstream and the T3 promoter sequence downstream. The purified plasmid DNA pre-digested with PvuII or VspI restriction endonucleases was used as template in the *in vitro* transcription. Run-off transcriptions was performed using T7 RNA polymerase (Fermentas UAB).

5.3. In-vitro *RNA editing reactions*

The *in-vitro* RNA editing reactions (Fig. 5.1) were performed in a total volume of 20 µl. The reaction mixture consisted of 30 mM HEPES-KOH pH 7.7, 3 mM magnesium-acetate, 45 mM potassium-acetate, 30 mM ammonium-acetate, 15 mM ATP, 2 mM dithiothreitol, 1% polyethyleneglycol 6000, 5% glycerol, 40 units RNase inhibitor (Fermentas UAB), 1 x proteinase inhibitor mixture (Complete TM, Roche Applied Science, Mannheim), 100 amol (100 x 10^{-18} mol) mRNA substrate, and 6.0 µl mitochondrial extract. After incubation at 28 °C for 4 h, the substrate mRNA was extracted with the RNeasy kit (Qiagen, Hilden).

5.4. Detection of the RNA editing activity by mismatch analysis

The cDNA was synthesized from the substrate mRNA with reverse transcriptase StrataScript (Stratagene, Heidelberg) from the T3 primer. The subsequent PCR reactions were performed with 0.1 units of Pwo polymerase (peqLab, Erlangen) and 0.5 units of Taq polymerase using a Cy5 labeled KS primer (Cy5-KS) and an unlabelled T3 primer. Cycling was performed as follows: 95 °C for 2 min; 5 cycles of touchdown PCR (95 °C for 30 s, 65 °C to 60°C decreasing by 1 °C per cycle for 30 s, and 72 °C for 1 min); 45 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min; finally 72 °C for 5 min. The PCR products were purified by 1% agarose gel electrophoresis.

Denaturation and reannealing were done as follows: 95 °C for 10 min; 90 °C to 70°C decreasing by 5 °C per cycle for 5 min; 65 °C for 1h. After reannealing, the resulting heteroduplexes were treated with 0.2 units of the enzyme TDG (thymine DNA glycosylase, Trevigen, Gaithersburg). The TDG-treated fragments were denatured for 5 min at 95 °C in alkali buffer (300 mM NaOH, 90% formamide and 0.2% bromphenol blue), and the strands of the DNA were separated by 6 M urea 6% PAGE. The Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (GE Health care, Freiburg). To quantify the efficiency of the *in-vitro* RNA editing reaction, the area under the peaks of the cleaved and uncut DNA fragments was determined. The ratio of cleaved, i.e. edited, fragment to uncut DNA was used to determine relative efficiencies of the investigated conditions in each experiment. To obtain comparable values to combine several independently repeated assays and to allow determination of variation bars, the ratios of cleaved to uncleaved fragments were displayed as percentages of the standard reaction conditions. Between individual experiments major sources of variation are the differences in RNA editing activity and RNase content of individual lysate preparations. In the gel analysis of the *in-vitro* editing products, smearing of the uncut fragment signal complicates the determination of the respective signal area for comparable quantification between individual gel runs. Therefore co-treatment and parallel resolution of the template under standard conditions was adopted for reference in each experiment.

The efficiency of detection has its optimum inside a window of a minimum editing percentage of 0,5 to 50%, and a substrate concentration of 100 amol using 60 to 120 µg of protein extract. Detecting two or more editing sites in one template, experimental detection inaccuracies have to be considered. The DNA template is labelled 5'. Every template, which contains more than one editing site can be edited at one or more sites in one molecule. The TDG-enzyme theoretically cuts every T / G mismatch, but only the labelled product will be detected. That way only the edited site which is situated closest to the labelled 5' end will be displayed.

5.5. Competition assays

Wild-type competitor RNA was synthesized from the PCR product amplified with primers T7 and +20 from clone *atp4*-40+20. 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 *atp4 CUCU –KS*, *atp4 UUCU –KS, atp4 UUCC –KS* with the T7 primer and primer +20. One hundred attomoles of substrate and 1,500 times competitor RNA (100 fmol) were first mixed and then incubated with the mitochondrial *in-vitro* assay as described above (Takenaka et al, 2004).

5.6. Standard methods of molecular genetics

All molecular genetic methods, e.g. enzymatic treatment of nucleic acids, electrophoresis etc. are performed according to the standard protocols commonly available.
Standard PCR reactions are performed using 10 µM of each primer in a 50 µl reaction. Buffer and Taq Polymerase BioTherm™ are provided by Genecraft GmbH (Lüdinghausen, Germany). For the dideoxy termination sequencing the Thermo Sequenase™ Primer Cycle kit (GE Health care) was used.

5.7. Materials

5.7.1. Consumables

Commercially obtainable chemicals of the companies Duchefa Biochemie B.V. (Haarlem, Netherland), VWR International GmbH (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), PEQLAB Biotechnologie GmbH (Erlangen, Germany), Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), Merck KGaA (Darmstadt, Germany), SERVA Feinbiochemica GmbH & Co. (Heidelberg, Germany) und MP Biomedicals GmbH (Eschwege, Germany). All buffers were prepared in double destilled water.

5.7.2. Enzymes

The enzymes used in this work were supplied by the companies Genecraft GmbH (Lüdinghausen, Germany), Fermentas GmbH (St. Leon-Rot, Germany), Roche Diagnostics GmbH (Mannheim, Germany), Stratagene GmbH (Heidelberg, Germany), and Trevigen, Inc. (Gaithersburg, USA).

5.7.3. Oligonucleotides

The oligonucleotides used in this work were commercially obtained from the companies biomers.net (Ulm, Germany) und Invitrogen GmbH (Karlsruhe, Germany). Sequences can be provided upon request.

5.7.4. Devices

The equipment used is standard of a Molecular Biological laboratory. For PCR the GeneAmp® PCR System 9700 of Applied Biosystems was used. For sequencing an ALF-Sequencer of GE Health care was used.

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7. Appendix

7.1 Publications

RNA editing sites in plant mitochondria can share cis-elements.

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RNA editing sites in plant mitochondria can share *cis*-elements

Johannes A. van der Merwe, Mizuki Takenaka*, Julia Neuwirt, Daniil Verbitskiy, Axel Brennicke

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

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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 a 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*-/*trans*-interactions can be effective over larger distances and can affect more than one editing event.
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Keywords: Plant mitochondria; RNA editing; Mitochondrial RNA

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 *atp9* 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 where 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 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 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).

*Corresponding author. Fax: +49 731 502 2626.
E-mail address: mizuki.takenaka@biologie.uni-ulm.de (M. Takenaka).

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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 uncut DNA fragments. The ratio of 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 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 invertion1 on the other. The resulting fragments were digested with *Eco*RI to generate sticky ends in the primer contained *Eco*RI 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 *atp9* 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 *atp9* 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 *atp9* 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 *atp9* 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 *atp9* 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

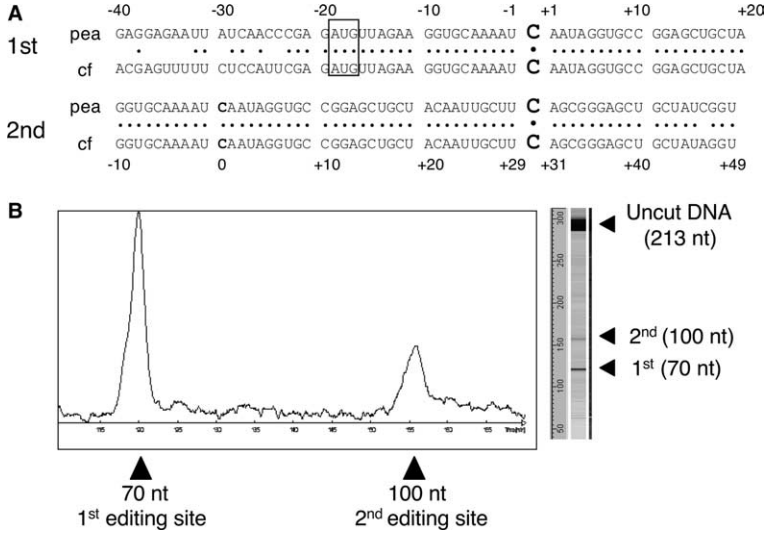


Fig. 1. Two neighbouring RNA editing sites are processed in an in vitro lysate from cauliflower mitochondria (*Brassica oleracea*). (A) Nucleotide sequence alignment of the first two editing sites (1st and 2nd) in the *atp9* mRNA reveals no sequence similarity around the sites. The edited C residues 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 *atp9* 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.

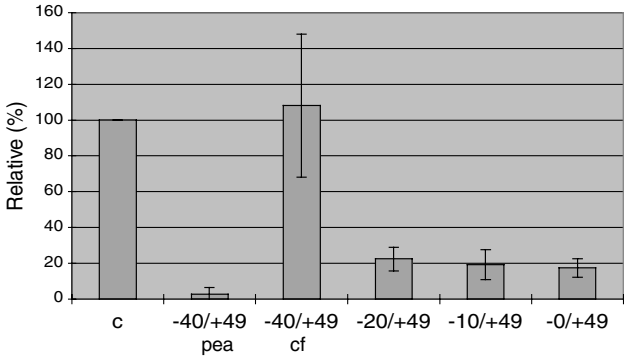


Fig. 2. Analysis of the *cis*-reqirements for in vitro RNA editing at the second site. The *atp9* sequences retained in the respective template RNAs are indicated underneath each column. All templates contain the second site at nucleotide +30 from the first site. In the three series of experiments summarized in this figure (five experiments for the cf −40/+49 template), editing at the first site in a pea template RNA covering −40/+49 is used as standard (column c). The deletion clones are derived from the pea template which is identical to the cauliflower sequence except for nucleotide +46. This difference 16 nucleotides downstream of the monitored second editing site is considered unlikely to be relevant for this editing event, since such downstream sequences are usually not involved in editing site definition.

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

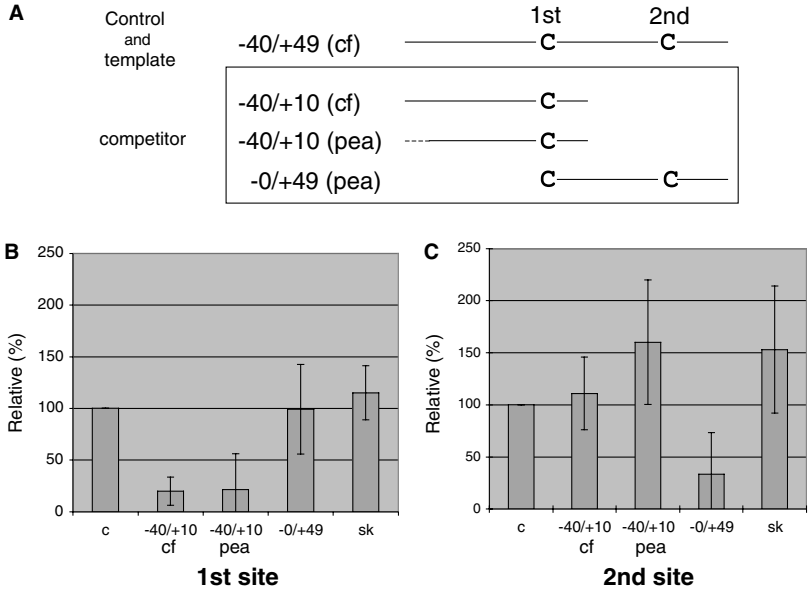


Fig. 3. Determination of competition for *trans*-factors between the respective core recognition sequences of the two neighbouring sites. (A) Scheme of the template *atp9* RNA from cauliflower (cf; top line), the competitors for the upstream site from cauliflower (second line) and from pea (third line), the dashed line indicating the different sequence in pea. The competitor for the downstream site (bottom line) differs only at nucleotide +46 between pea and cauliflower. The respective editing sites are indicated by a bold C. (B) The upstream site is monitored for effects by a 1500-fold excess of its upstream sequence (−40/+10) from cauliflower and pea, respectively, and of the downstream region (−0/+49), the latter containing the recognition sequence of the downstream site. The control c is run without competitor and the effect of a 1500-fold excess of a bacterial plasmid sequence is shown as sk. (C) The effect on in vitro editing of the downstream site by 1500-fold competitor excess of its own recognition sequence (−0/+49) and of the pea or cauliflower upstream site recognition sequences (−40/+10) is investigated.

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 targetted 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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Partially edited RNAs are intermediates of RNA editing in plant mitochondria.

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Partially edited RNAs are intermediates of RNA editing in plant mitochondria

Daniil Verbitskiy, Mizuki Takenaka*, Julia Neuwirt, Johannes A. van der Merwe and Axel Brennicke
Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany

Received 3 March 2006; accepted 11 April 2006.
*For correspondence (fax +49 731 502 2626; e-mail mizuki.takenaka@uni-ulm.de).

Summary

RNA editing in flowering plant mitochondria addresses several hundred specific C nucleotides in individual sequence contexts in mRNAs and tRNAs. Many of the *in vivo* steady state RNAs are edited at some sites but not at others. It is still unclear whether such incompletely edited RNAs can either be completed or are aborted. To learn more about the dynamics of the substrate recognition process, we investigated *in vitro* RNA editing at a locus in the *atp4* mRNA where three editing sites are clustered within four nucleotides. A single *cis*-element of about 20 nucleotides serves in the recognition of at least two sites. Competition with this sequence element suppresses *in vitro* editing. Surprisingly, unedited and edited competitors are equally effective. Experiments with partially pre-edited substrates indicate that indeed the editing status of a substrate RNA does not affect the binding affinity of the specificity factor(s). RNA molecules in which all editing sites are substituted by either A or G still compete, confirming that editing site recognition can occur independently of the actual editing site. These results show that incompletely edited mRNAs can be substrates for further rounds of RNA editing, resolving a long debated question.

Keywords: RNA editing, plant mitochondria, partially edited RNAs, *in vitro* RNA editing, atp4, cauliflower.

Introduction

RNA editing in plant mitochondria and chloroplasts is a post-transcriptional process altering mostly nucleotide identities from C to U in mRNAs and tRNAs. Previous *in vivo* and *in vitro* investigations found that for specific recognition by the RNA editing activity, often only about 20–40 nucleotides are necessary upstream and very few, if any, are necessary downstream of a given editing site (Bock *et al.*, 1996, 1997; Chaudhuri and Maliga, 1996; Choury *et al.*, 2004; Farré *et al.*, 2001; Hirose and Sugiura, 2001; Miyamoto *et al.*, 2004; Takenaka and Brennicke, 2003; Takenaka *et al.*, 2004).

The recent development of reliable *in vitro* activities for chloroplasts (Hirose and Sugiura, 2001; Miyamoto *et al.*, 2002) and mitochondria (Takenaka and Brennicke, 2003) has accelerated progress towards elucidating the details of *cis*-requirements, and has yielded further insights about the mode of editing site recognition.

For plant mitochondria, we have recently established *in vitro* RNA editing systems from pea shoots and from cauliflower inflorescences (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). Detailed analysis of the *cis*-requirements for site specificity showed that in some instances several elements can be distinguished in the *cis*-sequence of a given mRNA substrate (Neuwirt *et al.*, 2005). A basic essential region up to about 20 nucleotides upstream of an editing site in the *atp9* mRNA supports *in vitro* editing, albeit at a low level. A supporting region further upstream around the nucleotides from −35 to −40 enhances the *in vitro* RNA editing activity of this site.

Intriguingly the nucleotide identities in the immediate vicinity of the edited nucleotide have little influence on the specificity of recognition, and only moderately contribute to the efficiency of the reaction (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). The implied separation of recognition region and editing site may be bridged by an interconnected complex of several distinct proteins attaching to the different *cis*-elements in the RNA and the edited nucleotide respectively. Competition experiments suggest that the *trans*-factors for specificity can be titrated and are thus present in limiting quantities in plant mitochondria.

It is unclear, at present, how the editing machinery progresses along the substrate RNA. As in the steady state

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plant mitochondrial mRNA population a considerable percentage of transcripts is edited at most but not all sites, individual editing sites are most likely addressed independently of each other. *In vitro* analysis of RNA editing sites spaced 30 nucleotides apart has shown that each site is indeed addressed individually and substantiated this model (van der Merwe *et al.*, 2006). This however raises the question whether already edited sites can be discriminated from unedited sites, and whether both are seen as potential targets by the editing complex.

It has been an open question for many years whether incompletely edited RNA molecules, which contain some sites edited and others not edited, are either intermediates that can still be recognized as substrates to complete this process or whether such molecules represent dead ends that have been aborted and will be rapidly degraded (Bock, 2001; Bonnard *et al.*, 1992; Wissinger *et al.*, 1992).

To investigate whether the editing machinery can make this distinction between incompletely edited and not edited substrates, we analysed a cluster of three editing sites within four nucleotides in the *atp4* mRNA in unedited and incompletely edited states. Interestingly, one set of *cis*-elements mediates editing at the two prominent sites *in vitro* (and probably at all three *in vivo*) in the *atp4* mRNA.

Results

Three RNA editing sites are clustered within four nucleotides

In the *atp4* mRNA in cauliflower mitochondria three RNA editing sites are located within four nucleotides, raising the question of how these sites are addressed (Figure 1). *In vivo* the first and the third site appear to be slightly more rapidly edited: of 38 cDNA clones sequenced, 37 are edited at all sites, whereas one clone is edited only at sites one and three (data not shown). This observation, although statistically not significant, nevertheless suggests that *in vivo* site two is edited somewhat slower than are the other two sites. In terms of information content, site two is a silent editing as soon as site three has been altered, and mRNAs either unedited or edited at site two specify the same protein (Figure 1a). Looking at other plant species, most, including dicots, monocots and gymnosperms, require editing at site one, whereas only dicots code edited C residues at sites two and three (Figure 1b).

Such *status quo* analyses do not allow to distinguish between editing intermediates and terminally aborted errors in this processing step. We thus designed the respective *in vitro* experiments detailed below to determine whether incompletely edited molecules can still serve as substrates.

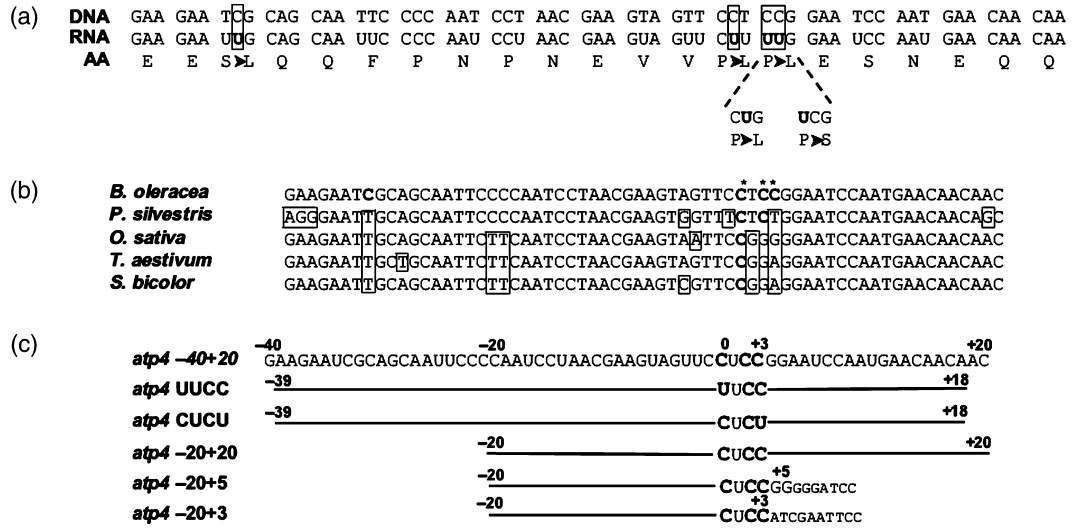


Figure 1. Three RNA editing sites are clustered within four nucleotides in the *atp4* coding sequence.
(a) Effect of RNA editing at the clustered sites on the encoded amino acid sequence. After editing the third site, the second site becomes a silent editing event, which does not change the amino acid specified. Uridines created by RNA editing are shown in bold and the resulting amino acid (AA) changes are indicated.
(b) Dicot plant species such as cauliflower (*Brassica oleracea*) contain a cluster of three editing sites, whereas monocots (*Oryza sativa*, *Triticum aestivum*, *Sorghum bicolor*) and gymnosperms (*Pinus silvestris*) require editing only at the first site. In the latter species editing at this site has not been experimentally shown. Nucleotide divergencies from the cauliflower sequence are boxed. The cluster of the three editing events in cauliflower is marked by asterisks. An additional upstream RNA editing event in cauliflower is indicated in bold and the consensus T at this position in other species is boxed.
(c) The initial substrate subcloned from the full length gene in cauliflower mitochondria contains 40 nucleotides upstream of the first edited C in the cluster, and 20 nucleotides downstream (*atp4 –40 + 20*; top line). Nucleotide positions are all numbered relative to the first editing site in the cluster, which is assigned position 0. Sequences of partially pre-edited RNA substrates as well as various deleted substrates examined for *in vitro* editing are depicted in the lines below. The three C-nucleotides edited *in vivo* as well as the respective edited U-moieties are shown in bold and larger type. Smaller fonts in the –20 + 5 and –20 + 3 deletion clones give the substituting bacterial sequences for comparison.

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In vitro *the first and the third of the three sites are edited*

In the cauliflower *in vitro* editing system sites one and three are altered correctly. The second site is not edited *in vitro* in line with the *in vivo* observation that this site may be somewhat less accessible to the editing machinery. Sequence analysis of individually cloned *in vitro* editing products suggest that editing sites one and three are altered independently of each other: of 20 analysed clones three were edited at both sites, whereas two were altered only at site one, and one was edited only at site three (data not shown). It remains unclear why the second site is not edited *in vitro*. We assume that *in vitro* not all necessary *trans*-factors assemble correctly for all sites, as several editing sites in different mRNA substrates we tested are not addressed *in vitro* (Takenaka M., Neuwirt J., van der Merwe J.A., Verbitskiy D., Zehrmann, Universität Ulm, Germany, unpublished observations). Similar observations were made in the *in organello* assays, in which several sites are never altered although these assays are closer to the *in vivo* situation than the *in vitro* lysates (Choury *et al*., 2004).

Twenty nucleotides upstream of the first site and two nucleotides downstream of the last editing site are sufficient for in vitro *recognition of both editing targets*

In vitro analysis with various deletion substrates (Figure 1c) reveals that upstream of the first site only twenty nucleotides are required for correct editing at both sites (Figure 2). The efficiency of editing is however lower than with a substrate containing 40 nucleotides upstream. This result suggests that the sequence context further upstream of the immediate 20 nucleotides supports the attraction of the *trans*-acting editing protein(s).

In the downstream region five nucleotides beyond the first editing site (i.e. two nucleotides beyond the 3′-terminal site) are sufficient for effective editing at both target nucleotides. Removal of a further two nucleotide identities does not inhibit editing at site one, but abolishes alteration at the 3′-terminal site three, suggesting that these downstream moieties are necessary for this latter event. In the substrate these two nucleotides were not actually deleted, but the GG dinucleotide was substituted by AT residues from the vector. We thus conclude that the two adjacent 3′-nucleotide identities are essential for editing at this position, but do not influence recognition of the first site located three nucleotides upstream.

Incompletely edited substrates are recognized as well as unedited sequences

To investigate the influence of the processing status of individual editing sites on recognition of this region, we constructed substrates in which the first and third sites were

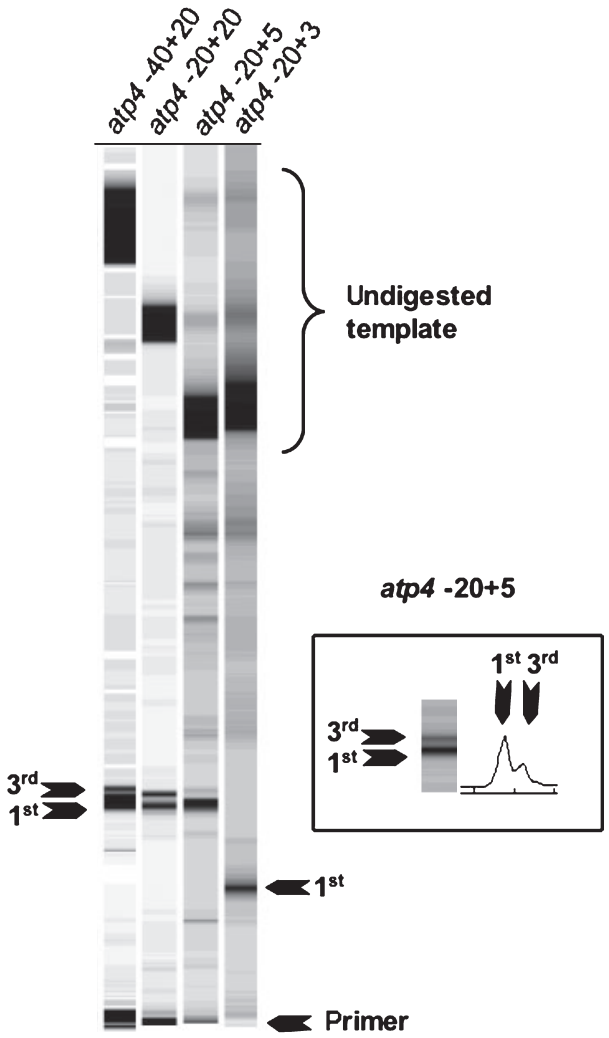


Figure 2. Determination of the minimal recognition site for the *atp4* editing site cluster. The longest substrate shown here (left lane, −40 + 20), covering 40 nucleotides upstream and 20 nucleotides downstream of the first editing site, is edited rather efficiently at both the first and third sites. The 5′-deleted substrate −20 + 20 with only 20 upstream nucleotides shows diminished editing activity at site 1. Deletion of downstream sequences in substrate −20 + 5 with only two nucleotides conserved downstream of the last site reduces the level of editing at both sites. Although only little *in vitro* editing product is seen at site three, the site as such is still recognized correctly. The inlet shows the gel image as well as the scan of a separate experiment with the −20 + 5 construct, in which the third site is more discernible. This 3′-most site is however not edited at all when the adjacent nucleotides downstream are substituted by different nucleotides in the −20 + 3 template (Figure 1c). The −40 + 20 and the −20 + 3 constructs are cloned in the *Sma*I site of the vector and the −20 + 3 TDG product is accordingly 20 nucleotides shorter. The −20 + 20 and −20 + 5 fragments are cloned into the *Eco*RV site of the vector, which is 18 nucleotides distant from the *Sma*I site. This results in TDG products with similar 5′-sequence extensions.

'pre-edited', i.e. already contained uridines at the respective positions. Both substrates are edited at the residual site with efficiencies comparable to the completely unedited substrate (Figure 3 and data not shown).

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Figure 3. Analysis of *in vitro* RNA editing in unedited and partially pre-edited substrates. The influence of processed versus unprocessed editing sites on the editing efficiency at respective other sites targeted by the same *cis*-element is investigated in a comparison of editing in an unedited mRNA substrate (left-hand lane, *atp4* C**U**C**C**−40 + 20), in an RNA edited at site 1 (center lane, *atp4* **U**U**CC**−39 + 18) and in a substrate edited at site 3 (right-hand lane, *atp4* C**U**C**U**−39 + 18). The signal of the third editing site in the completely unedited substrate appears to be weaker as a result of the action of the TDG enzyme, which in this majority of doubly edited products will render the downstream site undetectable when having cut at the first site. The unedited substrate RNA covers 61 *atp4* nucleotides, and includes each of the partially pre-edited substrates 58 nucleotides respectively. The TDG products of the latter substrate RNAs are 1 nucleotide shorter than the product from the unedited substrate, hence the corresponding shift in mobility. In the *in vitro* reaction of course only the respective residual and not the pre-edited site of the two potentially processed editing sites within this *atp4* region can be monitored. Sites of *in vivo* RNA editing are highlighted in bold as unedited **C** as well as editing product **U**.

To further evaluate the respective binding affinities we tested the effect of incompletely pre-edited, entirely unedited and fully pre-edited RNAs in competition with one another. In a first set of experiments competition against a substrate pre-edited at the 5′-most site, site one, was analysed for editing at site three (Figure 4). All unedited, pre-edited and fully edited RNAs compete to comparable effect. In a second round of experiments a substrate pre-edited at site three, the 3′-most site, was assayed for the effect of the various competitors on editing at site one (Figure 5). Also at this site all fully and pre-edited competitors inhibit *in vitro* editing to an effect comparable with the non-edited RNA. These results suggest that firstly the specificity mediating *trans*-factors for these RNA editing sites are present in limiting quantities, and secondly that these factors cannot discriminate between unedited and edited substrate RNAs.

Binding of trans-factors is independent of the editing site(s)

To further analyse the influence of the editing site upon recognition of the site by the *trans*-factors, we substituted the four nucleotides containing the three potential editing sites in the *atp4* template by continuous stretches of four As and Gs, respectively, and tested the effect of these RNAs as competitors (Figure 6). Both sequences inhibited the *in vitro* reaction at a wild-type template, suggesting that the limiting *trans*-factors bind upstream of the editing sites independently of either the presence or the absence of the actual edited nucleotide(s).

Specificity of the editing site recognition region

The nucleotide sequence of the specificity region for the three *atp4* editing sites investigated here is unique, at least in the *Arabidopsis* mitochondrial genome, and is different from the sequence contexts at other editing sites. To investigate this specificity, and the possibility that common *trans*-factors might recognize different sequences through some undetected common denominator in the vicinities of the various editing sites, we tested the effect of this *atp4* specificity region on RNA editing at two different editing sites in the *atp9* mRNA (Figure 7). None of the fully, incompletely or unedited *atp4* elements have any detrimental effect on editing at either of the two *atp9* sites *in vitro*. This result extends observations from several previous assays in mitochondria (and in plastids), which similarly suggested that indeed unique *trans*-factors recognize and bind to the various sequence elements around the approximately 30 plastid-located editing sites and the >400 RNA editing sites in plant mitochondrial RNAs (Chaudhuri *et al.*, 1995; Hirose and Sugiura, 2001; Miyamoto *et al.*, 2002, 2004; van der Merwe *et al.*, 2006).

Discussion

Incompletely edited RNAs can be substrates for RNA editing

The observation of incompletely as well as fully edited mRNAs in the steady state RNA population of plant mitochondria about 15 years ago has immediately raised the question of whether these incompletely edited RNAs are either terminally abandoned errors or whether they represent intermediates, in which RNA editing can still be completed (Hiesel *et al.*, 1990; Schuster *et al.*, 1990). This question has so far not been resolved as there was no experimental assay available to follow the fate of such

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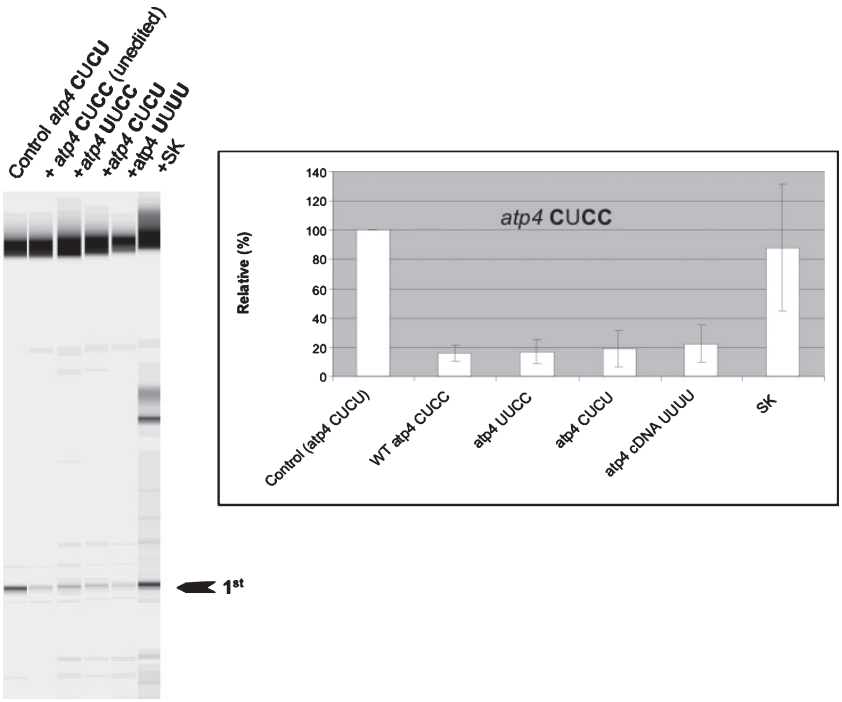


Figure 4. Competitor experiments of the first editing site with the *atp4* substrate region in various stages of RNA editing. The left-hand panel shows a representative gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the first site (arrowhead), the editing substrate was constructed to be an RNA in which the third site is already an edited **U** (*atp4* C**U**C**U**). The substrate reaction without competitor is run as a reference and set to 100%. The competitors (1000-fold) include an unedited sequence (*atp4* C**U**C**C**), partially pre-edited sequences altered at either the first site (*atp4* **UU**C**C**) or the third site (*atp4* C**U**C**U**), a fully pre-edited RNA with all three sites changed (*atp4* **UUU**C) and, as an unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from the bacterial competitor RNAs are detectable between the products from the full-length substrate and the TDG product after editing.

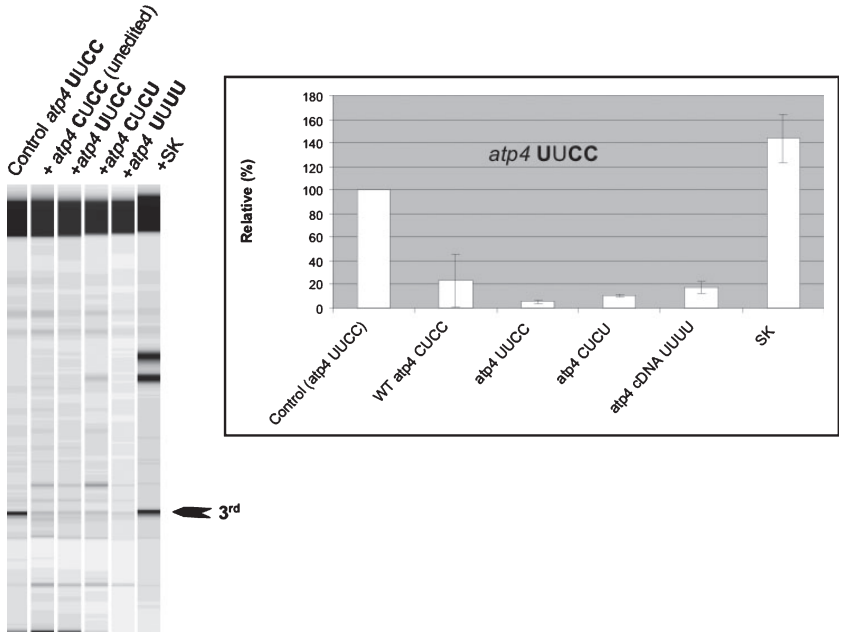


Figure 5. Competitor experiments for editing site three with the *atp4* substrate region in various stages of RNA editing. The left-hand panel shows a gel image, the bar graph on the right summarizes the results of three independent experiments. To monitor editing only at the third site, the editing substrate was constructed to be an RNA in which the first site is already a **U** (*atp4* **UU**C**C**). The substrate reaction without competitor is run as standard and set to 100%. Competitors (1000-fold) include an unedited sequence (*atp4* C**U**C**C**), partially pre-edited sequences altered at either the first site (*atp4* **UU**C**C**) or the third site (*atp4* C**U**C**U**), a fully pre-edited RNA with all three sites changed (**UUU**C) and, as unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full-length substrate and from the TDG fragment after editing.

incompletely edited molecules. The development of reliable *in vitro* RNA editing systems has now allowed us to address this long-standing debate and to provide a clear answer.

In the cauliflower *atp4* mRNA the constellation of three closely spaced sites offers a unique opportunity to investigate the processivity of the RNA editing complex in plant mitochondria, and to test whether its binding affinities are influenced by the editing status of the RNA substrate. In the direct approach incompletely edited substrates show comparable *in vitro* RNA editing at the respective unedited sites. The indirect assay, in which incompletely and fully pre-edited RNAs are employed as competitors, corroborates this result, as these molecules compete as effectively as the completely unedited RNA.

These experiments now show that incompletely edited RNA molecules can clearly act as competent substrates, and

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Figure 6. Competition between *atp4* RNAs, in which the four pyrimidines covering the three editing sites are substituted by either of the purines. To monitor editing only at the first site, the editing substrate was an RNA in which the third site is pre-edited to a **U** (*atp4* **U**U**CC**). The substrate reaction without competitor is run as standard and set to 100%. Competitors (1000-fold) are either one of the two purine sequences (*atp4* AAAA and *atp4* GGGG, respectively) or, as unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full-length substrate and from the TDG fragment after editing.

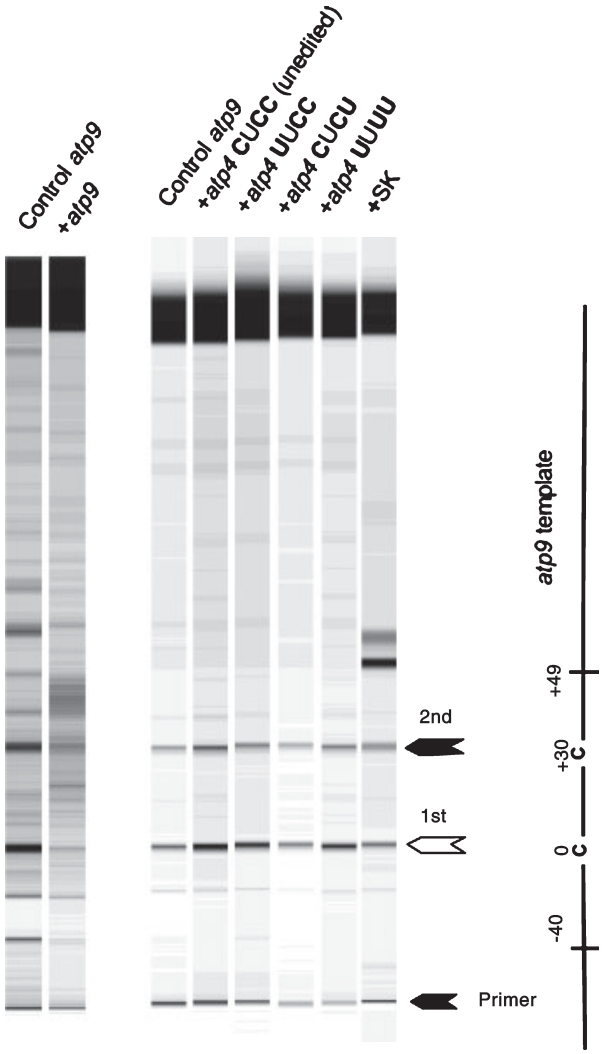


Figure 7. Heterologous competitor experiments to test the specificity of *trans*-factor binding. Two *atp9* editing sites located 30 nucleotides apart were monitored for the effect of 1000-fold competitors of the *atp4* substrate region in various stages of RNA editing. The locations of the two *atp9* RNA editing sites (first and second) are indicated in the schematic of substrate RNA (*atp9*) in the right-hand margin. The substrate reaction without competitor is run as a reference and control. Competition with the homologous RNA blocks RNA editing almost completely at each of the two sites, whereas heterologous RNA editing site sequences have little effect (quantification of the gel data not shown). Heterologous competitors include an unedited *atp4* sequence (*atp4* **C**U**CC**), partially pre-edited sequences altered at either the first site (*atp4* **UUCC**) or the third site (*atp4* **C**U**CU**), a fully pre-edited RNA with all three sites changed (**UUUU**) and, as an unspecific and unrelated control, a bacterial sequence (SK). RT-PCR products inadvertedly amplified from this bacterial competitor RNA are detectable between the products from the full-length substrate and the TDG product after editing.
Sequence data accession numbers: DQ202504.

thus have to be considered as intermediates in the processing step of RNA editing. This result furthermore makes it unlikely that these intermediates could give rise to a group of slightly variant proteins with modified properties in plant mitochondria (Grohmann *et al.*, 1994; Lu and Hanson, 1994; Phreaner *et al.*, 1996). The more probable scenario is that functional proteins will only be specified by fully edited RNAs as supported by protein sequence analysis (Grohmann *et al.*, 1994; Lu and Hanson, 1994).

Specificity determinants do not include the nucleotide(s) to be edited

The observation that the editing activity binds to unedited as well as edited RNA molecules suggests that the *trans*-acting specificity factors do not recognize the nucleotide to be edited. In the special situation of three clustered editing sites in the *atp4* mRNA, a single upstream *cis*-region acts as anchor for all these sites, *in vitro* at least for the two sites correctly addressed, as editing of both sites is lost upon the deletion of nucleotides closer than 20 nucleotides upstream (data not shown). The editing complex does however

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recognize the identity of the nucleotides downstream of the last editing site for contact at this site, but must also keep the C, which is to be edited, accessible for contact by the actual editing enzyme(s). This latter conclusion is confirmed by the efficient competition of RNA molecules in which the four pyrimidines covering the three editing sites are substituted by either purine. These experiments suggest that the identity of the to-be-edited nucleotide has no influence on the recognition of the specific editing site by the *trans*-acting factor(s).

Two scenarios can alternatively explain these observations: In the first, the editing enzyme itself is not sequence-specific and is guided to an editing site by the separate specificity factor(s). These latter factors do not contact the nucleotide to be edited, but allow the enzyme to approach the RNA and to perform the editing reaction. The sequence-specific binding proteins can then additionally contact the downstream nucleotide(s), but must leave a gap for the approach of the enzyme. On the other hand the editing enzyme itself could have some steric preferences that require certain nucleotide identities in this downstream region. However, considering the large number of editing sites in plant mitochondria with very different downstream nucleotides, this latter constraint appears to be less likely. In the alternative scenario, instead of an assembly of several proteins with these distinct (partial) functions, a single *trans*-acting protein may contain a respective enzymatic from C to U deamination domain, and there may not be a separate editing enzyme. This single protein would have to recognize the specificity region, bind to the RNA without regard to the editing status of either this or other editing sites and attempt to alter the target nucleotide.

Site-specific distance requirements between the cis-recognition element and the nucleotide to be edited

In this region in the *atp4* RNA, two cytidines separated by two nucleotides are addressed from the same specificity region. This suggests that the editing complex assembled here has at least this three-nucleotide tolerance and a correspondingly low constraint on the distance from the specificity region. This flexibility is high in comparison to an editing site in the *atp9* RNA, which cannot be shifted by a single nucleotide in either direction (Neuwirt J. and Takenaka M., Universität Ulm, Germany, unpublished results). These observations suggest that not only different proteins assemble at the respective *atp9* and *atp4* editing sites, as predicted by the different sequences of the *cis*-elements, but also that these complexes have variant properties at least with respect to the flexibility of the outreach of the editing activity to the respective nucleotides to be edited.

The heterologous competition experiments between the *atp4* and *atp9* regions show that the *trans*-factors for these sites are indeed different and specific, as the heterologous *atp4* editing specificity region has little influence on editing at the respectively monitored sites in the *atp9* RNA. This observation supports the conclusion that although the specificity factor(s) are limiting in their abundance in plant mitochondria, the enzyme(s) performing the actual deaminating step from C to U are available in much higher concentrations. The alternative scenario is not excluded by these results, in which single protein moieties recognize and alter a given editing site, so that deletion of one would not affect the performance of the other RNA editing activities.

A single specificity factor targeting several editing sites in dicot plants and evolutionary aspects

Only dicot plants, such as cauliflower, genomically encode the three C-nucleotides found edited *in vivo* in the cauliflower *atp4* mRNA (Figure 1a,b). Monocot plants such as either rice or maize only code the first C, the other nucleotides have different identities and thus are not subject to editing. The gymnosperm *Pinus silvestris* does code for the first two Cs, but the third site is already genomically encoded as T. This makes the second editing event obsolete because it does not change the respective codon identity. Monocots and gymnosperms should thus both possess at least the *trans*-acting specificity factor to recognize the first editing site. This factor may have evolved in dicot plants to be able to recognize three sites. Alternatively, additional *trans*-factors may be guided to the editing complex, which could support editing at either one or the other additional sites. The observation of independent editing at sites 1 and 3 in our *in vitro* assays would be explained either by such additional co-factors or by slightly variant editing enzymes. A hypothetical co-factor for the central site in dicots could be present in very low quantities in our assays, which would explain why we do not observe this site edited *in vitro*. Alternatively, assuming only one specificity factor (plus the enzymatic moiety), editing at this second site may require previous editing at the other positions, sites one and three. To investigate this possibility we tested a substrate pre-edited at sites one and three in the *in vitro* assay (data not shown). However, we still did not observe any editing at site two suggesting steric hindrance in the artificial substrate RNA, the requirement for an additional co-factor, or a long temporal lag, which pushes effective editing at this site outside the range of the *in vitro* incubation time.

The scenario of only one specific *trans*-acting moiety targeting all three (two *in vitro*) editing sites, and thus coupling the editing events, is supported by the relative numbers of partially edited clones from the *in vitro* reaction (data not shown). The identified three clones edited at both sites one and three are, although few, still many more than predicted from a completely independent association of several additional factors, as only two clones edited at site one and one clone edited at site three were observed in the

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total of twenty clones analysed. This distribution suggests that a bound *trans*-factor, which processes site one, is already in position and is thus more likely to also process site three (and vice versa). This is most likely explained by a single binding factor that can stretch to either or both sites.

The comparison of RNA editing in this region between the different plant species raises the question whether the editing systems in monocot and gymnosperm mitochondria can edit all the three sites found in dicot plants. Such an investigation could be pursued for example in the *in organello* RNA editing systems developed for wheat and maize mitochondria (Choury *et al.*, 2004; Farré *et al.*, 2001). If these sites are correctly processed in monocots, we could conclude that plant mitochondria have the potential for many more RNA editing sites than those that have been realized.

Experimental procedures

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 PercoII gradient (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). Isolated mitochondria (400 mg) 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 a 30-min incubation on ice, the lysate was centrifuged at 22 000 ***g*** for 20 min. The supernatant was recovered and dialysed 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^{-1}) was rapidly frozen in liquid nitrogen.

RNA substrates

Genomic as well as cDNA sequences for the *atp4* coding region in cauliflower were determined to identify RNA editing sites. The sequence data are deposited in the databases under accession number DQ202504. DNA clones (p*atp4*) were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing substrate RNA as previously described (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). Substrate RNAs were synthesized from the T7 RNA polymerase promoter and thus contained vector sequences at the 5′-end. Similarly vector sequences border the 3′-end of the mitochondrial insert sequences (Figure 1c) up to the *Vsp*I site used for linearization of the template DNA. The bordering bacterial sequences were used for specific amplification of the substrate RNAs by RT-PCR after the *in vitro* assay. Mutant RNA competitors with purines instead of the native pyrimidines were synthesized from PCR products with respectively modified primer sequences.

Deletion clones were shortened by removing original mitochondrial sequences as indicated in the respective experiments. The 5′-deletion mutants were constructed by inverted PCR from p*atp4* with respective primers pairing to the −40 and −20 upstream sequences on the one side and primer invertion1 on the other. The resulting fragments were digested with *Eco*RI to generate sticky ends in the primer-contained *Eco*RI recognition site and were self-ligated. The outside bacterial anchors for PCR amplification moved accordingly 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.

In vitro *RNA editing reactions*

The *in vitro* RNA editing reactions were performed as described (Neuwirt *et al.*, 2005; Takenaka and Brennicke, 2003). After incubation, substrate sequences were amplified by RT-PCR, the upstream primer being labelled with the Cy5 fluorophor. RNA editing activity was detected by mismatch analysis employing the TDG enzyme activity (thymine DNA glycosylase; Trevigen, Gaithersburg, MD, USA). The TDG-treated fragments were separated and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (Amersham, Freiburg, Germany).

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 cleaved, i.e. edited, fragment to uncut DNA was used to determine the 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 with a co-treated substrate.

Competition assays

Competitor RNAs were synthesized by T7 RNA polymerase as run-off products from the PCR products amplified with primers T7 and respective primers matching the different *atp4* 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. Substrate (100 attomol) and 1000 times (100 fmol) competitor RNA were first mixed and then incubated with the mitochondrial *in vitro* assay as described above. In the competitor RNAs either the KS or the T3 sequences were deleted to avoid contaminating PCR amplifications of the monitored substrate RNAs.

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RNA editing in plant mitochondria does not require energy

RNA editing in plant mitochondria does not require energy

Mizuki Takenaka, Daniil Verbitskiy, Johannes A. van der Merwe, Anja Zehrmann, Uwe Plessmann*, Henning Urlaub* and Axel Brennicke

Molekulare Botanik, Universität Ulm, 89069 Ulm, Germany;
*Max Planck Institute for Biophysical Chemistry, Bioanalytical Mass Spectrometry Group, Am Fassberg 11, 37077 Göttingen, Germany

Correspondence author:
Dr. Mizuki Takenaka
Molekulare Botanik
Universität Ulm
89069 Ulm, Germany

e-mail: mizuki.takenaka@uni-ulm.de
Tel: + 49-731-502-2616
Fax: + 49-731-502-2626

Coauthors e-mail: Daniil Verbitskiy, daniil.verbitskiy@uni-ulm.de ; Anja Zehrmann, anja.zehrmann@uni-ulm.de ; van der Merwe, dre.vandermerwe@uni-ulm.de ; Axel Brennicke, mo.bo@uni-ulm.de ; Uwe Plessmann, uplessm@gwdg.de ; Henning Urlaub, henning.urlaub@mpi-bpc.mpg.de

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Keywords: RNA editing, plant mitochondria, glutamate dehydrogenase, RNA-binding, in vitro RNA editing, cauliflower

1. Introduction

In plants RNA molecules are in both organelles altered by RNA editing. In plastids of flowering plants about 35 and in mitochondria about 400 selected cytosines are changed to uridines. The direct biochemical effect of RNA editing in these plants is thus a site-specific deamination. Despite years of investigation neither the reaction mechanism nor the enzymes involved have been identified. Several clues on - or rather conditions of - the biochemistry of the reaction have emerged mostly from in vitro analyses of plastid and mitochondrial lysates respectively. The first in vitro assays of plant mitochondrial lysates suggested that the sugar-phosphate bonds of the affected RNA molecule are not disrupted in the polynucleotide chain [1]. This observation excludes insertional editing which would excise and exchange either the nucleotide or the base. Thus either direct deamination or transamination are the most likely mechanisms. The first reaction would per se require no added energy, while for the latter, the transamination reaction, additional molecules of higher molecular energy would most likely be involved.

For mitochondrial lysates a strict requirement for added ATP has been observed [2]. Similarly in plastids additional ATP has been reported to be required in most lysates [3-5], while in some residual activity can be seen without any added ATP [6,7]. Surprisingly the added ATP can be substituted to full effect by some or all of the other NTPs and even dNTPs [2,6,7]. While all of these molecules are virtually interchangable in mitochondrial lysates, they vary in their effect in plastid extracts [6,7]. In both organelles at least one of the dNTPs is as effective as ATP, suggesting that one of the few enzymes accepting either triphosphate is involved. One group of such enzymes is a class of RNA helicases and their participation has consequently been proposed [2].

We have now investigated the requirement for nucleotide triphosphates in detail and come to the surprising conclusion that this dependence is at least in mitochondria almost entirely explained by the behaviour of the enzyme glutamate dehydrogenase (GDH).

2. Materials and methods

2.1. Preparation of mitochondrial extracts

Cauliflower mitochondria were purified by differential centrifugation steps and a Percoll gradient as described [2,8]. Isolated mitochondria were lysed, the lysate was cleared and the supernatant was recovered and dialyzed as detailed previously [2,8].

2.2. RNA substrates

DNA clones were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing substrate RNA as described [2,8]. Substrate RNAs containing vector sequences at the 5'- and 3'-ends of the mitochondrial insert sequences were synthesized from the T7 RNA polymerase promoter in the linearized template DNA. The bordering bacterial sequences were used for specific amplification of the substrate RNAs by RT-PCR after the in vitro assay [9,10]. The ^{32}P-labelled RNA templates for the gel shift assays were obtained by direct incorporation of labelled ATP into the RNA during run-off transcription from the DNA template.

2.3. In vitro RNA editing reactions

The in vitro RNA editing reactions were performed as described [8,11]. After incubation, substrate sequences were amplified by RT-PCR, the upstream primer being 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 and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (GE Healthcare).

2.4. Protein identification

RNA editing templates were labelled with biotin (biomers GmbH) and 500 pmol were incubated with 100 µl of mitochondrial lysate in a total volume of 200 µl under standard in vitro RNA editing conditions [2]. The proteins in contact with the RNA were cross-linked by UV irradiation for 20' (UV Stratalinker 1800, Stratagene). The biotin-labelled RNA-protein complexes were bound to streptavidine sepharose™ High performance (GE healthcare). Unbound proteins were washed off by six rinses in spin columns with 400 µl wash buffer each (30 mM HEPES-KOH pH 7.7, 3 mM magnesium acetate, 45 mM potassium acetate, 30

mM ammonium acetate, 10% glycerol). The sepharose beads were treated with RNase A to release the RNA-bound proteins, which were collected with wash buffer.
Proteins with their bound residual RNA fragments were analysed by PAGE and/or were (directly) identified by liquid chromatography coupled (LC) electrospray ionization (ESI) tandem mass spectrometry (MSMS). Proteins cross-linked to RNA were digested within the gel using trypsine according to Shevchenko et al. [12]. For digestion of proteins in solution after extraction from the beads, the samples are denatured in 4 M urea and 2 M thiourea, diluted to a final concentration of ≤ 1M urea and incubated with 2 µg trypsine overnight at room temperature. Peptides are desalted and separated by nanoLC equipped with a pre-column working in backflush and directly analyzed by ESI MSMS in a Q-ToF (Q-ToF ultima, Waters) or a hybrid triple quadrupole/linear iontrap mass spectrometer (4000 QTrap, ABI) under standard conditions. Fragment spectra of peptides are searched against the NCBInr database using Mascot as search engine. Matches with resulting protein identification were usually obtained against the *Arabidopsis thaliana* sequences, which is phylogenetically closely related to the here analysed cauliflower (*Brassica oleracea*). Since different species are compared, similarities/differences in the respective orthologous protein sequences additionally influence the generated scores.

3. Results and Discussion

3.1. NTP as well as dNTP support the in vitro RNA editing reaction

The strict requirement for added NTP in the in vitro RNA editing reaction is documented in figure 1. No RNA editing product with uridine in the relevant position of RNA editing is detectable if no NTP is added. Addition of ATP at the optimal concentration of 15 mM yields about 2-5 % C to U converted nucleotide, the amount depending upon the lysate, the source of the mitochondria and the template used. The positive effect of ATP can be fully recovered by substitution with CTP, shown as example here (Fig. 1). Any of the NTPs or dNTPs can replace ATP and will yield comparable editing rates [2]. Since in plastid as in mitochondrial in vitro systems NTPs and dNTPs can substitute for ATP, analogous factors may be responsible for the in vitro ATP requirement in both organelles.

3.2. Influence of the added NTP on template RNA-protein interactions

To investigate the protein moieties affected by the added NTP/dNTP in their ability to contact and bind to the RNA substrate molecules, we performed gel shift experiments with radioactively labelled RNA editing substrates (Data not shown). Comparisons of the protein patterns cross-linked with the template RNA in the presence or absence of ATP on PAGE-gels and by more sensitive mass spectroscopy (MS)-analyses did not reveal any qualitative differences. The overall quantity of the proteins labelled with the crosslinked RNA, however, changes dramatically with the presence or absence of NTP: Without added ATP the same proteins are labelled much stronger (Data not shown). This suggests that addition of ATP disturbs all protein-RNA interactions non-specifically.

3.3. Identification of proteins bound by the RNA editing template

Since the gel shift experiments did not yield detectable proteins differentially affected by NTP in their affinity to the RNA editing template, we employed a more sensitive affinity purification scheme to identify respective polypeptides. This procedure achieved an enrichment of the proteins bound to the editing template RNA (Fig. 2). The Coomasie-stained PAGE patterns of proteins retained by the RNA template in the presence or absence of ATP, however, again showed only little qualitative difference and no clear candidate protein could be assigned by its ATP-dependent affinity to the template in this gel analysis (Data not shown).

Because the ATP-dependence of the in vitro reaction may not manifest in abundant specific RNA-protein interactions and the PAGE analysis may not be sensitive enough, we proceeded to analyse the set of proteins interacting with the RNA directly by the much more sensitive MS without prior gel separation. The proteins bound by the RNA editing template were hydrolyzed and peptides were identified by liquid chromatography (LC) coupled tandem MS (MSMS) analysis. To identify the proteins, the obtained fragment spectra were searched against all entries in the NCBInr database. Since cauliflower and *Arabidopsis thaliana*, which latter is completely sequenced, are closely related and the protein sequences of many household enzymes are to a large extent identical, we should be able to identify cauliflower proteins through their orthologues in *Arabidopsis thaliana*. The proteins contained in the total complement enriched by the affinity purification procedure and identified by conserved peptide sequences indeed yielded a list of mitochondrial proteins from *Arabidopsis thaliana* (Table 1).

Within this list we focussed on proteins which might be involved in a deaminase or transaminase reaction. Among the general mitochondrial proteins such as heat shock proteins, malate dehydrogenase and such, some of the more prominent proteins were representatives of the glutamate dehydrogenase group of proteins, i.e. GDH1 and GDH2 (Table 1) [13]. GDH can catalyze amination as well as deamination reactions depending on the presence and concentration of various allosteric and isosteric regulators (Fig. 3). There is thus a (albeit remote) possibility that one or more of these enzymes have been recruited into the RNA editing reaction to catalyse the deamination step from C to U.

Furthermore GDH-proteins have been previously identified as RNA binding proteins [14] and have been investigated particularly with respect to their participation in RNA editing in kinetoplasts of trypanosomes. Simpson and coworkers showed that the preferential binding of GDH to guide RNA is fortuitous and not related to RNA editing [15]. However, in kinetoplasts RNA editing involves the insertion and deletion of specific uridines rather than the deamination events found in mitochondria and plastids of flowering plants.

3.4. Is the GDH involved in the RNA editing reaction?

To investigate a potential participation of the GDH in the C to U deamination reaction, we tested the influence of various cofactors of this enzyme on the in vitro editing assay. The most prominent and essential cofactors of the normal GDH catalyzed reactions are NADH and NADP for the deamination and amination reactions, respectively. NAD, NADP and their respective reduced forms NADH and NADPH were individually added to the in vitro RNA editing reaction. The assay also contained the suboptimal concentration of 10 mM ATP to be able to observe either enhancing or detrimental effects. NADH as well as NADPH stimulated the reaction while the oxidized dinucleotides NAD and NADP did not (Data not shown). Since only enhancing effects were detected, the next series of assays investigated the effect of NADH and the other dinucleotides in the absence of ATP to see whether they can substitute at least partially for the mononucleotide. Both NADH and NADPH were able to replace the NTP requirement, while NAD and NADP did not support the in vitro RNA editing reaction (Fig. 4).

3.5. Investigation of the role of the GDH in the in vitro RNA editing assay

This result of NADH and NADPH being able to substitute for NTP suggests that NTP may not be required for the RNA editing reaction per se, but may be required to alleviate the inhibitory effect of the GDH by releasing this protein from the template RNA or by keeping it away a priori. To test this hypothesis that the GDH might be a major inhibitor of the in vitro RNA editing activity, we analysed the effect of a direct blocker of the GDH proteins.

The compound phosphinothricin is such a specific inhibitor of the GDH in plants and binds irreversibly to the active site of the enzyme. Addition of phosphinothricin to the in vitro RNA editing reaction indeed recovered the editing activity in the absence of any added NTP (Fig. 5). This result shows that NTP solely serves to keep the GDH protein(s) away from the template and that no added energy is required for the RNA editing reaction per se.

The equivalent capability of NADH and the other reduced dinucleotide NADPH suggests that the site of action of these compounds is the GDH itself. Whether the added NTPs and dNTPs also bind to the GDH remains open.

If the NTPs and dNTPs do not directly interact with the GDH, the NTP/dNTP and the NADH/NADPH/phosphinothricin effects could be achieved by different modes of action: While the first may activate an RNA helicase [2] which removes the inhibiting bound GDH, the latter compounds may have the same final effect by directly blocking the GDH. The ultimate result observed with both sets of added chemicals, namely active in vitro RNA editing, could be the outcome from either mechanism. To decide this question, the effect of added (d)NTPs and NAD(P)H on the RNA binding of the GDH will have to be investigated with the purified GDH enzyme. The observation that all unspecific RNA-binding proteins in a mitochondrial extract are diminished upon addition of ATP to the gel-shift or cross-linking reaction (Data not shown) suggests that the NTP/dNTP effect is indeed not a direct action on the GDH, but rather a separate general RNA-wiping effect mediated by an RNA helicase. Nevertheless, the substitution of NTP by phosphinothricin shows that if a helicase is stimulated by the added NTP/dNTP, the activity of this helicase is not essential for the actual RNA editing reaction, but at least in vitro mainly serves to remove the inhibitory GDH.

3.6. RNA editing does not require added NTP in vitro

The observed in vitro RNA editing of different template RNAs (Data not shown) in the absence of added NTP suggests that the actual biochemical reaction does not consume energy. The in vitro observed requirement of added NTPs, dNTPs, NADH or phosphinothricin may

not be relevant in the in vivo situation: in the intact mitochondrion, RNA editing as well as splicing and 5'- and 3'-processing most likely occur compartimentalized and in safe distance from the location of the GDH.

In chloroplast extracts in vitro RNA editing is similarly stimulated by or depends on the addition of NTP or dNTP moieties. Here the plastid located NADPH-dependent GDH may play an analogous inhibitory role and corresponding specific inhibitors such as phosphinothricin should be tested to resolve this question. It has been reported however that neither NADH nor NADPH have any effect on the in vitro reaction, which may argue against an analogous role [6]. On the other hand the effects of individual NTP and dNTP identities vary between extracts from different plant species [3,6,7].

The correct in vitro RNA editing in a plant mitochondrial lysate in the presence of GDH-inhibiting phosphiniothricin shows that the GDH is not involved in the deamination of C to U although this enzyme regularly catalyzes an analogous reaction and can bind RNA molecules. These conclusions make a direct deamination process by a modified cytidine deaminase or an analogous enzyme more probable than a transaminase reaction, for which a requirement of some additional source of activation energy would be more likely - although energetically not strictly necessary.

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Figure legends

Fig. 1. In vitro RNA editing of an *atp4* mRNA template requires the addition of ATP or CTP. Similar to the in vitro editing of an *atp9* RNA [2], dNTP-nucleotides can substitute for the NTP in this reaction (Data not shown).

Fig. 2. Cauliflower proteins binding to the atp9 RNA editing template were purified by crosslinking to the RNA. The *atp9* RNA editing template contains 40 nucleotides upstream and 10 nucleotides downstream of the first editing site. Proteins were spread on an analytical PAGE-gel and silver-stained. The arrow indicates the position of the GDH as determined from the molecular weight of the *Arabidopsis thaliana* protein(s). It is assumed that the cauliflower protein analysed here has a comparable molecular weight.

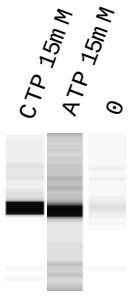
Fig. 3. The reactions catalyzed by the glutamate dehydrogenase include deamination and amination steps. The GDH enzymes bind NAD(P) or NAD(P)H for the activities of amination and deamination, respectively. The direction of the dominant reaction is influenced by the levels of ATP. It is possible that one of these or a similar enzyme has mutated to access also RNA molecules and deaminate specific cytidines.

Fig. 4. NADH and NADPH can substitute the requirement for NTP in the in vitro RNA editing reaction. These assays monitor the first editing site in an *atp4* template RNA in which no other site can be edited since the adjacent downstream nucleotides have been altered [9]. The lane marked 0 contains no added (di)nucleotide in the control in vitro incubation. The lanes marked CTP, NADH, NAD, NADP, NADPH contain 15 mM of the respective compounds in the in vitro reaction mix. The gel image shown is the relevant portion of the fluorescent detection of the Cy5 labelled RT-PCR products after TDG treatment, which recognizes the mismatches at the T moieties introduced by in vitro editing.

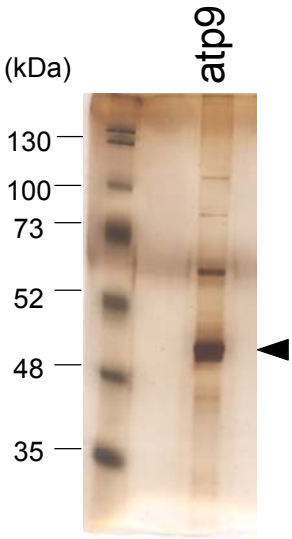
Fig. 5. The GDH-specific inhibitor phosphinothricin can substitute the requirement for NTP in the in vitro RNA editing reaction. All assays monitor the first editing site in an *atp4* template RNA. In the control in vitro incubation no nucleotide was added (lane marked 0). The lanes marked “phosphinothricin” contain this compound but no added NTP or dNTP in the in vitro reaction mix. Only the relevant portion of the gel image is shown.

Fig. 6. Schematic overview of the GDH interference with in vitro RNA editing. The GDH proteins have access to added RNA molecules in the mitochondrial lysate where internal compartimentations of the mitochondria are disrupted. Binding of the GDH to RNA inhibits access of the RNA editing trans-factors to the template RNA. The GDH is kept from the template RNA by blocking the RNA-binding site with either NAD(P)H or with the specific inhibitor phosphinothricin (or any of the NTP/dNTP nucleotides; upper part). Alternatively the GDH is removed from the RNA by an RNA helicase which is activated by either NTP or dNTP, thus giving access to the trans-factors of RNA editing (lower part).

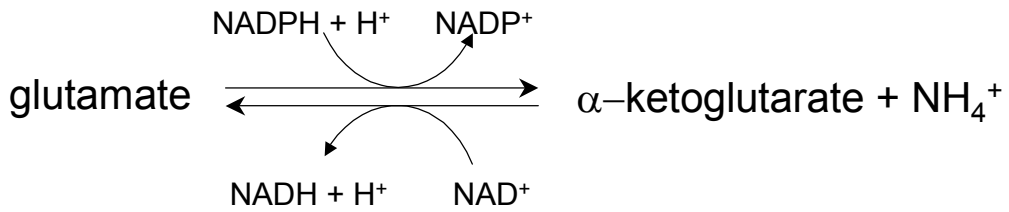
Table 1. Typical protein identification results of an MS analysis after enrichment and UV-crosslinking of cauliflower mitochondrial proteins binding to the *atp9* RNA editing template. The orthologous proteins from *Arabidopsis thaliana* with relevant scores listed here are those expected in plant mitochochondria and previously identified in proteome analyses [16].



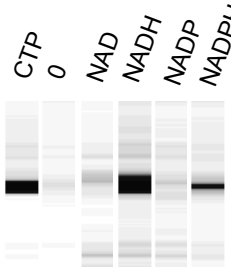
Takenaka et al Fig 1



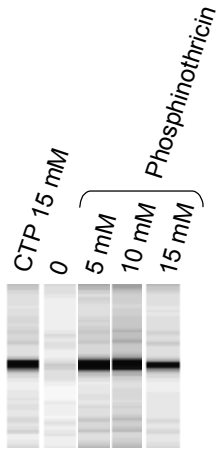
Takenaka et al Fig 2



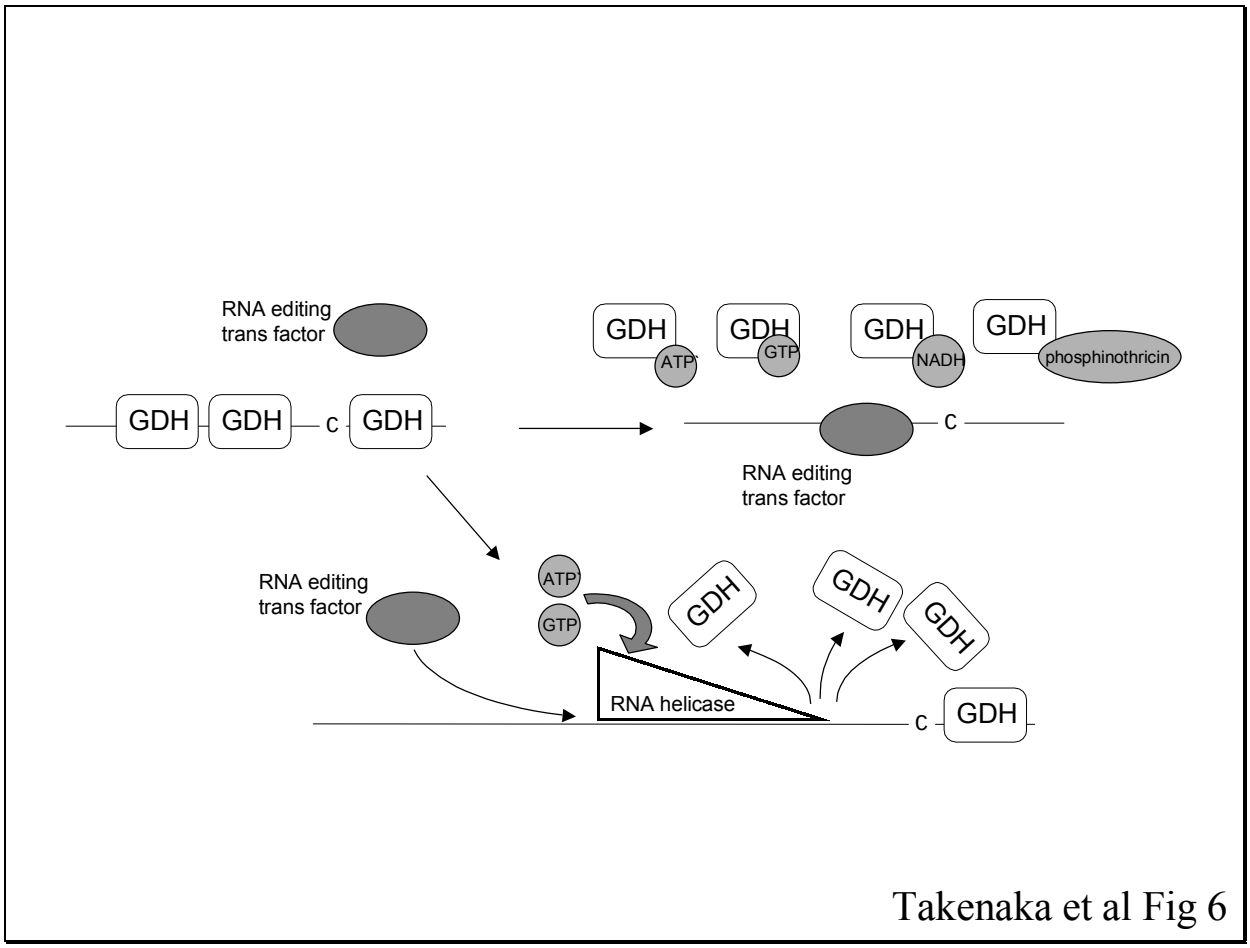
Takenaka et al Fig 3



Takenaka et al Fig 4



Takenaka et al Fig 5



Takenaka et al Fig 6

Table 1 Proteins identified as binding to the atp9 RNA editing template in cauliflower mitochondrial extracts

Gene identifier	Mass	Score	Queries matched	Name
gi|4210330	116581	839	19	2-oxoglutarate dehydrogenase, E1 subunit
gi|7076784	114147	575	12	2-oxoglutarate dehydrogenase, E1 subunit-like protein
gi|2654210	72330	489	9	heat shock 70 protein
gi|17939849	63332	435	7	mitochondrial F1 ATP synthase beta subunit
gi|15238762	44496	324	7	GDH1 (GLUTAMATE DEHYDROGENASE 1); oxidoreductase
gi|520478	39163	258	4	pyruvate dehydrogenase E1 beta subunit
gi|4210332	49908	226	7	2-oxoglutarate dehydrogenase E2 subunit
gi|415733	62316	210	4	mitochondrial chaperonin
gi|15240793	44671	191	4	GDH2 (GLUTAMATE DEHYDROGENASE 2); oxidoreductase
gi|710400	43003	134	5	pyruvate dehydrogenase E1 alpha subunit
gi|6435320	25248	123	3	nucleoside diphosphate kinase

The table shows the typical protein identification results of a MALDI analysis after enrichment of cauliflower mitochondrial proteins binding to the atp9 RNA editing template by UV-crosslinking. The orthologous proteins from *Arabidopsis thaliana* with relevant scores

listed here are those expected in plant mitochochondria and previously identified in proteome analyses (Millar *et al*, 2005).

7.2. Curriculum vitae

Name:	***Verbitskiy,Daniil***	
Adress:	Donaustrasse 8 89073 Ulm	
Date of birth:	19.08.1973	
Place of birth:	Krasnodar/Russia	
Nationality:	Russia	
Marital status:	single	
Education:	9. 1980 – 5. 1990	Middle school in Irkutsk/Russia
	9. 1990 – 6. 1995	study of biology at the University of Irkutsk/Russia main subjects: molecular biology, genetics,
	2. 1993 – 6. 1995	"Diploma work" in the Laboratory of Dr. Slobodjanuk Sergey, department of gene systematic at the Limnological institute of SAR Irkutsk/Russia **subject:** "BspMII type of satellite DNA of the Bikal cottoids and their phylogeny."
	9.1995-1.2004	junior research worker at the laboratory of the genetic engineering of plants, SIPBP SAR institute, Irkutsk/Russia
	from 3. 2004 on	PhD with Prof. Dr. Axel Brennicke, department of molecular botany, University of Ulm **subject:** "Recognition of the *atp4* editing cluster"
languages:		Russian, English,

7.3. List of Publications

Papers:

van der Merwe JA, Takenaka M, Neuwirt J, Verbitskiy D, Brennicke A. RNA editing sites in plant mitochondria can share *cis*-elements. FEBS Lett. 2006 Jan 9;580(1):268-72.

Verbitskiy D, Takenaka M, Neuwirt J**,** van der Merwe JA and Brennicke A. Partially edited RNAs are intermediates of RNA editing in plant mitochondria. Plant J 2006, 47(3): 408-416

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Reviews

Takenaka M, van der Merwe JA, Verbitskiy D, Neuwirt J**,** Zehrmann A, Brennicke A. RNA editing in plant mitochondria. Version 4. 12. 2005, Review for Goeringer book, in press.

Posters

2005 Poster at the RNA Editing Gordon research conference in Ventura: "*In-vitro* analysis of *cis*-and *trans*-elements for RNA editing in plant mitochondria" Takenaka, M; Neuwirt, J; van der Merwe, JA; Verbitskiy, D and Brennicke, A.

2005 Poster at the "International congress on plant mitochondria biology" in Obernai/France: "*In-vitro* analysis of *cis*- and *trans*-elements for RNA editing in pea and cauliflower mitochondria" Takenaka, M; Neuwirt, J; van der Merwe, JA; Verbitskiy, D and Brennicke, A.

2005 Poster at the 3rd Japanese-German joint symposium: "*Cis*- and *trans*-requirements for RNA editing in plant mitochondria." Takenaka, M; Neuwirt, J; van der Merwe, JA; Verbitskiy, D and Brennicke, A.

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Erklärung

Hiermit versichere ich, dass ich vorliegende Arbeit selbstständig angefertigt habe und keine anderen Hilfsmittel, als die angegebenen benutzt, sowie wörtlich und inhaltlich übernommene Stellen als solche kenntlich gemacht habe.

Ulm, im April 2007

Daniil Verbitskiy