In Vitro RNA Editing in Pea Mitochondria Requires NTP or dNTP, Suggesting Involvement of an RNA Helicase*

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To analyze the biochemical parameters of RNA editing in plant mitochondria and to eventually characterize the enzymes involved we developed a novel in vitro system. The high sensitivity of the mismatch-specific thymine glycosylase is exploited to facilitate reliable quantitative evaluation of the in vitro RNA editing products. A pea mitochondrial lysate correctly processes a C to U editing site in the cognate atp9 template. Reaction conditions were determined for a number of parameters, which allow first conclusions on the proteins involved. The apparent tolerance against specific Zn²⁺ chelators argues against the involvement of a cytidine deaminase enzyme, the theoretically most straightforward catalyst of the deamination reaction. Participation of a transaminase was investigated by testing potential amino group receptors, but none of these increased the RNA editing reaction. Most notable is the requirement of the RNA editing activity for NTPs. Any NTP or dNTP can substitute for ATP to the optimal concentration of 15 mM. This observation suggests the participation of an RNA helicase in the predicted RNA editing protein complex of plant mitochondria.

RNA editing has been observed in various forms in diverse organisms including trypanosomes, mammals, slime molds, and land plants (1). In land plants, RNA editing has so far been reported in mitochondria and plastids. In flowering plants the majority of this organelar editing involves C to U conversions, with U to C changes occurring much more infrequently and only in mitochondria (2, 3, 4). In non-flowering plants this latter direction of RNA editing is much more common in mitochondria as well as in chloroplasts, the U to C reactions almost reaching the frequency of the C to U editing in the hornworts (5). Since its discovery more than a decade ago, progress into understanding how this process works has been hampered by the lack of manageable and reliable in vitro systems. The first in vitro system for plant mitochondrial RNA editing was successfully developed from wheat embryos (6), but has not been exploited further.

Conclusions about the determinants of specificity during RNA editing in mitochondria and in plastids have initially been drawn from comparisons of transcribed sequence duplications (e.g. 7). Such comparisons suggested that upstream (5') sequences are crucial in targeting the editing machinery and that downstream similarities are not sufficient to specify a site.

These observations were corroborated by the recent breakthrough development of an in vitro editing system for plastids (8, 9) and an electroporation protocol for mitochondria (10, 11). Mutational analysis of templates in these as well as in vivo experiments in transgenic chloroplasts (12–14) showed that for several editing sites 20–30 nucleotides upstream and 2–5 nucleotides downstream are sufficient to guide the editing activity, precisely what had initially been deduced for mitochondria (7).

The biochemistry of this RNA editing activity is still largely unclear, and more detailed understanding requires accessible in vitro systems for experimental investigations. Initial in vitro experiments from plant mitochondria had indicated that the sugar phosphate backbone remains intact during the deamination step of C to U editing (6, 15–17). Analogous observations with the in vitro systems for tobacco and pea chloroplasts further support the similarity between the editing processes in both organelles (8, 9). The most likely enzyme able to catalyze this reaction could be one of (or a homologue of) the cytidine deaminases identified in plants (18) as well as in other organisms. One of these deaminases is in mammals the crucial enzyme of the apolipoprotein mRNA C to U editing (19). Problematic for this group of deaminases, however, appears to be the reverse reaction, because none of the homologous enzymes from various organisms has been found to be able to catalyze the amination step leading to a U to C conversion. The recent identification of this class of enzymes in Arabidopsis thaliana will allow us to determine their potential involvement in RNA editing (18).

To facilitate direct investigations into the biochemistry and specificity of RNA editing in plant mitochondria we have now developed an in vitro system from pea mitochondria, which allows comparatively rapid and robust access to biochemical parameters. This system employs the advantages of mismatch detection and incision in double-stranded DNA by a thymine DNA glycosylase (TDG)1 enzyme (reviewed in Ref. 20). We report ion and cofactor requirements of pea mitochondrial RNA editing which suggest participation of an RNA helicase but do not support the involvement of a classic cytidine deaminase.

EXPERIMENTAL PROCEDURES
RNA Substrates—DNA clones (patp9) were constructed in an adapted pBluescript SK+ to allow run-off transcription of the editing template RNA. The synthesized RNA molecule contains the first two pea atp9 editing sites flanked by bacterial sequences to allow specific amplification against the background of internal mRNAs (Fig. 1). 154 bp of the 5′ untranslated region and the first 69 bp of the coding sequences of the pea mitochondrial atp9 gene are cloned into the PstI site of the vector multiple cloning site between the T7 promoter followed by a KS sequence on the upstream side and the T3 promoter sequence in the downstream region. Downstream of the T3 sequence follow 89 bp

1 The abbreviations used are: TDG, thymine DNA glycosylase; nt, nucleotide; RT, reverse transcription.

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* This work was supported by a grant from the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of the mRNA 3’ terminal double stem-loop sequence of the pea mitochondrial atp9 gene flanked by the 20 upstream and 22 downstream adjacent nucleotides. To facilitate easier cloning, 6 nucleotides are added at the 5’ flank to create an NdeI recognition site and at the 3’ end 3 additional nucleotides generate a PvuII site (Fig. 1). The RNA substrate for the in vitro editing reaction is transcribed as a run-off product from PvuII-digested patp9 using T7 RNA polymerase (MBI).

The resulting transcript (475 nt) was denatured and the single strands of the DNA were separated by 6 M urea 6% formamide 0.2% bromphenol blue. After reannealing, the resulting heteroduplexes were treated with 0.2 units of the enzyme TDG (thymine DNA glycosylase, Trevigen). The resulting transcript is 69 nt upstream and 183 nt downstream sequences outside of the atp9 region. The upstream part corresponds to vector sequences from the T7 transcription start point to the PvuII restriction site. The downstream anchor consists of vector sequences from the PvuII site to the T3 promoter region and the downstream attached pea atp9 double stem-loop sequence.

Preparation of Pea Mitochondrial Extracts—Pea seedlings (Pisum sativum L., var) were grown at 25°C in the dark for 6 days. Mitochondria were isolated by differential centrifugation and purification on Percoll gradients as described previously (21). Four hundred mg of purified mitochondria were lysed in 1,200 ml of 100 mM potassium-acetate, 30 mM magnesium-acetate, 45 mM potassium-acetate, 30 mM ammonium-acetate, 15 mM ATP, 2 mM dithiothreitol, 1% polyethylene glycol 6000, 5% glycerol, 40 units RNase inhibitor (MBI), 1X proteinase inhibitor mixture (Complete™, Roche Applied Science), 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 RNAeasy kit (Qiagen). Variations of individual concentrations and additions of various other compounds are indicated in the respective figure legend.

Detection of the RNA Editing Activity by Mismatch Analysis—The mRNA substrate was transcribed into cDNA with the reverse transcriptase StrataScript (Stratagene) from the T3 primer. The subsequent PCR reactions were performed with 0.1 units of Pwo polymerase (peqLab) 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 rennealing 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 1 h. After rennealing, the resulting heteroduplexes were treated with 0.2 units of the enzyme TDG (thymine DNA glycosylase, Trevigen). The TDG-treated fragments were denatured for 5 min at 95°C in alkaline buffer (300 mM NaOH, 90% formamide, and 0.2% bromphenol blue), and the single 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 (Amersham Biosciences).

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 lysozyme preparations. In a gel analysis of the in vitro editing products, sequencing of the uncleaved fragment signal complicates the determination of the respective signal area for comparable quantification between individual gel runs. Therefore cotreatment and parallel resolution of the template under standard conditions was adopted for reference in each experiment.

RESULTS

The in Vitro Editing System from Pea Mitochondria—To investigate the biochemical mechanisms of RNA editing, we developed an in vitro system based upon the pea atp9 mRNA as a model substrate and a mitochondrial lysate from etiolated pea seedlings. Preparation of the mitochondrial lysate essentially followed the steps of the protocol for an RNA editing active lysate from tobacco chloroplasts (8, 9). Mitochondria prepared from 6-days-old pea seedlings as detailed under “Experimental Procedures” were lysed with 0.2% Triton X-100 in the presence of 2 mM KCl. Mitochondrial debris was pelleted by centrifugation and the resulting protein supernatants were extensively dialyzed.

The pea mitochondrial atp9 gene was chosen as standard substrate because this gene is abundantly transcribed and has been investigated in detail with respect to its promoters and transcript stability (22, 23). It is furthermore efficiently edited at most of its editing sites, including the first site in the reading frame, which was selected as the editing target. The general applicability of the system was probed with a second RNA template, which covers part of exon e from the nad5 gene in the pea mitochondrial genome (data not shown).

The Detection System—Briefly, plasmid patp9 was constructed to generate the mRNA substrate for the in vitro reaction (Fig. 1). The run-off RNA transcribed from the T7 promoter in the PvuII-digested patp9 plasmid serves as the 475 nt substrate that is incubated with the pea mitochondrial extract. In this RNA 173 nt upstream of the first editing site and 49 nt downstream are genuine atp9 sequences. A second editing site lies 30 nt downstream of the first and is thus covered by the cloned region. Flanking this continuous mitochondrial sequence are (beyond the leftovers from the multiple cloning site) the sequences of the T3 and the transcribed part of the T7 phage RNA polymerase promoters, respectively, integrated. The T3 promoter sequence and the KS sequence downstream of the residual T7 promoter serve as specific PCR primer binding sites. Downstream adjacent 89 nt from the pea atp9 mRNA 3’ terminus are inserted, which contain the stability conferring inverted repeat region (23).

S. Binder, personal communication.
After incubation in the lysate, total RNA is isolated for RT-PCR between primers Cy5-KS and T3, which are specific to the bacterial anchor inserts in patp9 and do not match any internal sequence of plant mitochondria (Fig. 1). The resulting DNA fragments are denatured and slowly reannealed, partially forming heteroduplexes at heteroplastic sites. Nucleotide variants are expected at the sites of RNA editing, where a certain percentage of all molecules of template RNA and consequently of the PCR products will contain U respectively a T, whereas most molecules retain the unedited C. The majority of the G-containing antisense strand will result in a respective majority of T/G mismatched molecules.

These heteroduplexes are detected as cleaved DNA fragments resulting from a TDG enzyme treatment (Fig. 2). TDG specifically recognizes T/G and G/G mismatches in heteroduplexes and nicks one of the two DNA strands at these sites, in the TG mismatch the strand with the T and in the G/G mismatch either of the two. Because primer KS is labeled with Cy5, the cleaved fragments can be detected as shorter single-stranded DNA molecules in an automated DNA sequencer. An exemplary image of the automated DNA sequencer analysis of stranded DNA molecules in an automated DNA sequencer. An

**Fig. 2. Detection and documentation of the in vitro RNA editing activity.** A, gel image of a TDG detection analysis on an ALF sequence machine. B, electropherograms of the sequencing lanes. The peak at 218 nt corresponds to the TDG fragments cleaved at the first editing site, the peak at 248 nt represents the second site fragments. C: control reaction with an artificial (1:1) mixture of patp9 and patp9c DNAs reveals the expected size of the TDG fragments. Plasmid patp9c (in which atp9 cDNA was cloned instead of genomic atp9 DNA) differs from plasmid patp9 only at the two editing sites, where patp9c contains the edited Ts rather than the genomic Cs. +Ex, RNA template incubated with mitochondrial extract. –Ex, RNA template incubated without mitochondrial lysate.

these clones showed C to U conversion at site 2, which explains the absence of a detectable signal at this site in the TDG detection assays. Because the putative cis-signals for this second site should occur here as well. An experiment aimed at this second site by deletion of the upstream site likewise showed no editing, and more detailed specific experiments are required to investigate why this site is not altered by the lysate.

**Extract Concentration, Time Course, Optimal Temperature, and RNA Substrate Quantity**—The in vitro editing activity showed a linear increase with increasing concentrations of extract up to adding 60–120 μg of protein extract to the final volume of 20 μl (Fig. 4A). Similar to observations made with the chloroplast lysate, tests with various extract preparations

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showed high protein concentrations to be crucial for activity. At saturating extract concentrations, the amount of edited product increased linearly up to 4 h of incubation time, showing that the lysate retains its activity during this time (Fig. 4B). Evaluation of the temperature influence on the in vitro editing reaction revealed a fairly sharp optimum at 28 °C with little activity observed at 37 °C, one of the temperature extremities investigated (Fig. 4C and data not shown).

Tests of various amounts of the RNA substrate showed that 100 amol supply enough template to clearly detect the editing reaction. Lower quantities of the substrate tended to favor unspecific RT-PCR amplifications (data not shown), whereas higher amounts of the substrate yielded less efficient RNA editing. Thus 100 amol were routinely used in all further assays (Fig. 4D). Depending on the individual lysate preparation 60–120 μg mitochondrial protein extract and 4 h incubation time at 28 °C were adopted as standard conditions.

Ion Requirements of the RNA Editing Activity—Several standard ions were investigated at varying concentrations to learn more about the properties and requirements of the editing enzyme(s) and to determine optimal concentrations. For K⁺ ions efficient editing of the RNA substrate was observed at levels between 20 and 45 mM (Fig. 5A and data not shown). Further increasing the concentration of this salt to 50 mM resulted in inhibition of the activity. With CaCl₂ strong inhibition was observed already at a concentration of 5 mM (Fig. 5B). Increasing concentrations of Mg²⁺ inhibited the editing reaction less prominently. Titration of Mg²⁺ ions against the concentration of ATP to compensate the effect of the phosphate seems not crucial, because increasing Mg²⁺ ions beyond 3 mM at 15 mM ATP inhibited the reaction (not shown). The sensitivity to Ca²⁺ ions is possibly due to activation of unspecific protease(s) in the crude mitochondrial fraction employed here, which may degrade essential protein components of the RNA editing activity. Similarly, Mg²⁺ ions possibly activate unspecific nucleases in the extract. In a wheat mitochondrial in vitro RNA editing system (6) optimal Mg²⁺ ion concentrations were found to be 10 mM, higher than those observed here in pea, whereas K⁺ salts had no effect up to 150 mM in the wheat system (6). These differences possibly reflect distinctions between the different plant species analyzed. In subsequent assays K⁺ was adjusted to 45 mM and Mg²⁺ to 3 mM, whereas no further Ca²⁺ ions were added.

NTP/dNTP Dependence of the RNA Editing Reaction—Because for the chloroplast in vitro RNA editing reaction Hirose and Sugiiura (8) had reported a prominent influence of ATP with an optimum at 3 mM, we investigated the effect of the ATP concentration on the RNA editing activity in the mitochondrial system. The pea mitochondrial lysate displays an even higher requirement for added ATP in that no editing is observed without at least 5 mM ATP, the optimal concentration of ATP being reached only at 15 mM (Fig. 6A). The editing activity does not plateau with higher ATP concentrations, but rather is inhibited by further increasing levels of this compound. This is again different from the wheat mitochondrial lysate, where no additional ATP was required (6).

We then investigated the influence of other NTPs and dNTPs and surprisingly find that any NTP or dNTP can substitute for ATP in the editing reaction (Fig. 6, B and C). Mixed assays show that the effect of different NTPs is cumulative. Addition of 5 mM of either CTP, GTP, or UTP to a 10 mM ATP reaction mixture increases the editing activity to about the level of 15 mM ATP (Fig. 6D, +5 mM and data not shown). Addition of 15 mM of any of these NTPs to 10 mM ATP results in a distinct decrease of the editing activity analogous to the one observed with 25 mM ATP alone (Fig. 6D, +15 mM and data not shown). Thus we conclude that the reaction can use any of the NTPs or dNTPs as essential cofactor in the reaction, with the optimal NTP or dNTP concentration being 15 mM in this assay (Fig. 6).

Zinc Requirements of the RNA Editing Reaction and Effect of Chelators—The specific Zn²⁺ chelator 1.10-phenanthroline and the nonspecific divalent ion chelator 1.7-o-phenanthroline show comparable influences on the efficiency of the RNA editing reaction. Both had similarly little effect at 1 mM, inhibited the reaction somewhat at 5 mM and completely blocked editing at 25 mM respective concentrations (Fig. 7 and data not shown). These observations suggest that no free Zn²⁺ ions are required for the reaction, although of course internally bound Zn²⁺ ions may be protected from this interference.

The inhibitory effect of divalent ion chelators was furthermore investigated by addition of EDTA or EGTA to the in vitro reaction (Fig. 8). Both chelating compounds inhibit the reaction, the editing activity is almost completely lost at 50 mM...
EDTA or EGTA, respectively. This further confirms that divalent ions are required, which can be titrated by chelators, but we can as yet not deduce their identity.

**Tests for Cofactors as Potential Acceptors for the Amino Group**

Several compounds used in transamination reactions in other biochemical pathways were tested for their influence on the RNA editing reaction.

**FIG. 4.** Influence of amounts of mitochondrial extract, incubation time, temperature, and concentration of RNA substrates on the editing reaction. *A*, the *in vitro* editing reaction was carried out with varying K-acetate concentrations revealing an optimum around 20–45 mM. *B*, incubation at different CaCl₂ concentrations showed that more than 1 mM interfere with the *in vitro* RNA editing. *A–B*, After incubation, RT-PCR, TDG treatment, and electrophoresis the ratios of digested and undigested fragments are plotted as detailed for Fig. 4. Conditions used routinely in *vitro* are 45 mM K-acetate and no added CaCl₂.

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**FIG. 5.** Influence of K⁺ and Ca²⁺ concentrations on the editing reaction. *A*, the *in vitro* editing reaction was carried out with varying K-acetate concentrations revealing an optimum around 20–45 mM. *B*, incubation at different CaCl₂ concentrations showed that more than 1 mM interfere with the *in vitro* RNA editing. *A–B*, After incubation, RT-PCR, TDG treatment, and electrophoresis the ratios of digested and undigested fragments are plotted as detailed for Fig. 4. Conditions used routinely in *vitro* are 45 mM K-acetate and no added CaCl₂.

with various amounts of mitochondrial (mt) extract. A concentration of 6 μl in a total volume of 20 μl was found to be sufficient and was henceforth used. *B*, different incubation times showed an optimum of the edited product to be reached after about 240 min. In each of two experiments this incubation time is set as standard and the other signals are plotted as relative percentages. For these the variation between two independent experiments is indicated by bars. An incubation time of 4 h was then routinely used. *C*, the optimal incubation temperature for the *in vitro* editing reaction was found to be about 28 °C, which was subsequently employed. In the bar graph the relative percentages of the editing signal are shown at the other temperatures and the variation between two experiments is indicated. *D*, various amounts of RNA substrate were tested in the *in vitro* editing reaction. The highest percentage of edited fragments was observed with 0.1 fmol (100 amol). Adding only 10 amol (0.01 fmol) yielded mostly unspecific RT-PCR amplification products (data not shown). The ratios of digested and undigested fragments are plotted for each incubation condition as relative percentages of the standard (usually the optimal) condition in each experiment. Variation between two separate assays is indicated where this was possible.
on the RNA editing activity (data not shown). However, neither glutamate, H9251-ketoglutarate, nor aspartate changed the efficiency of the editing reaction. Actually, these low molecular compounds should have been completely eliminated from the extract by the extensive dialysis. Addition should have increased the editing reaction, if any of these compounds was active as amino group acceptor in a transaminating reaction.

**DISCUSSION**

**The in Vitro System**—We here report a sensitive in vitro system for RNA editing in plant mitochondria and its application to investigations of molecular and biochemical characteristics of this process. Such an open system should allow conclusions about the enzymatic activities involved. Our novel detection system identifies in vitro RNA editing events via PCR amplification of cDNA and mismatch detection consistently and reproducibly. We find the high sensitivity of the detection cascade to be essential for a robust mitochondrial in vitro system, because the efficiency of the in vitro activity is not very high. From the direct sequence analysis of 400 cDNA clones as well as from the observed signal strength in the mismatch system we estimate about 3–4% of the cytosines at the investigated atp9 site to be deaminated to uridines in an average in vitro reaction. In the previously established in vitro system for wheat mitochondria, the wheat atp9 gene was edited to about 6%, a similarly low efficiency (6). In their chloroplast in vitro system Hirose and Sugiura (8) observed about 10–30% editing of the added template RNA, 5–8 times more efficient than the wheat (6) or our pea mitochondrial extracts.

All the variations of mitochondrial extract preparations we tested did not further improve the efficiency, and we are tempted to think that this limitation maybe an inherent feature of mitochondria. Possibly the more than 10-fold higher number of different RNA editing sites in mitochondria versus...
chloroplasts in flowering plants dilutes essential factor(s) to reaction-limiting concentrations. Indeed any attempt at stretching the amount of extract used per experiment rapidly leads to a complete loss of editing (Fig. 4A and data not shown). This suggests that the concentrations of one or more compounds in the mitochondrial lysate are crucial to get any editing at all.

**In Vitro RNA Editing at Another Site**—To evaluate the generality of the *in vitro* system, we tested in addition to the *atp9* template also an RNA substrate covering exon e of the *nad5* gene in pea (data not shown). The selected region was cloned into the arrangement described for *atp9* (Fig. 1). The generated run-off RNA contains four RNA editing sites, the most 5’ of which was investigated *in vitro*. *In vitro* editing is found to alter the correct nucleotide also in this template (data not shown), confirming the mitochondrial extract to contain all necessary factors for editing at several sites.

**Involvement of an RNA Helicase?**—ATP requirement, which is usually equal to energy needs, can yield important information about the enzyme(s) involved in a biochemical reaction. For the chloroplast *in vitro* RNA editing reaction, Hirose and Sugiura (8) reported a strict ATP independence. Furthermore, a non-hydrolyzable analog was not able to substitute for ATP, suggesting that ATP is indeed used as an energy source. In our plant mitochondrial *in vitro* system the RNA editing activity is similarly dependent on and influenced by the concentration of ATP (Fig. 6). Conversely in the wheat mitochondrial *in vitro* system (6) addition of nucleoside triphosphate did not affect RNA editing, which may be due to different biochemical requirements between monocot and dicot plants or a feature of the different lysate preparation protocols.

In our pea system the optimal concentration of ATP was observed at 15 mM, beyond which higher concentrations inhibited the editing activity. Surprisingly any of the other NTPs or dNTPs can substitute for ATP in the editing reaction, which is not very common for enzymatically catalyzed reactions. Some of the few enzymes accepting NTP or dNTP belong to a subgroup of the RNA helicases (24, 25). RNA helicases have been identified in plants through *in silico* analyses of the genomic and cDNA data in the databases and a number of putative genes have been found in the *A. thaliana* genome (26). Several of these are predicted to contain N-terminal pre-sequences, which may target the respective protein to either the chloroplast and/or the mitochondrion. These predictions, however, need to be investigated experimentally in each instance. So far, evidence for only one RNA helicase as a mitochondrial enzyme has been published, AtSUV3, the homologue of the yeast *suv3* gene (27). Mitochondrial subcellular targeting and ATPase activity have been shown for this protein, but its general NTP requirements have not yet been tested in detail. In yeast the *suv3* enzyme is potentially involved in RNA turnover and its homologue in plants may have a similar function. This analogy would suggest that one of the other predicted mitochondrial helicases could be a more likely candidate helicase for RNA editing.

**Involvement of a Cytidine Deaminase?**—The deamination of cytidine to uridine typical for higher plant mitochondria and chloroplasts could most easily be achieved by a direct deamination reaction such as those catalyzed by cytidine deaminases. The most prominent example of a cytidine deaminase diverted to RNA editing is the modification of the apolipoprotein B mRNA in several mammals (1, 19, 28, 29). To investigate the potential participation of such a cytidine deaminase, we tested the RNA editing activity in our plant mitochondrial extract for its sensitivity to chelators of Zn$^{2+}$ ions. Such specific chelators can completely block the classic cytidine deaminases including the apobec-1 enzyme, which requires Zn$^{2+}$ ions to deaminate a single C in the apolipoprotein B mRNA (28, 29).

The insensitivity of the pea mitochondrial editing reaction to the Zn$^{2+}$-ion-chelator suggests the involvement of an enzyme distinct from the classic cytidine deaminases. Indirect support for this conclusion also comes from the occurrence of reverse reactions in plant mitochondria (i.e., the C to U deamination reactions), which have never been found to be catalyzed by a cytidine deaminase. Enzymes known to mediate both forward and reverse reactions include transaminases, such as those involved in amino acid synthesis pathways.

The possibility of a transamination reaction was tested by monitoring the effect of several potential NH$_2$ acceptors used in transamination reactions *in vitro*, glutamate, α-ketoglutarate, and aspartate. None of these potential acceptors improved the efficiency of the editing reaction. A transamination reaction is nevertheless possible with different amino group acceptors, because the biochemical and enzymatic requirements for the C to U deamination as well as the U to C aminating reaction would most parsimoniously be explained by a single transaminase acting for both directions of the amino group transfer.

**Acknowledgments**—Special thanks to Drs. Masahiro Sugiura, Tetsuya Miyamoto, and Junichi Obokata for their advice and kind communication of unpublished results. We also thank Dr. Stefan Binder for his constructive comments and suggestions. The excellent technical support of Dagmar Pruchner is gratefully acknowledged.

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