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Involvement of a site-specific *trans*-acting factor and a common RNA-binding protein in the editing of chloroplast mRNAs: development of a chloroplast *in vitro* RNA editing system

Tetsuro Hirose[1,2] and Masahiro Sugiura[1,3,4]

[1]Center for Gene Research, Nagoya University, Nagoya 464-8602, Japan

[2]Present address: Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, 295 Congress Avenue, BCMM 133, New Haven, CT 06536, USA
[3]Present address: Graduate School of Natural Sciences, Nagoya City University, Nagoya 467-8501, Japan

[4]Corresponding author
e-mail: sugiura@nsc.nagoya-cu.ac.jp

RNA editing in higher plant chloroplasts involves C→U conversion at ~30 specific sites. An *in vitro* system supporting accurate editing has been developed from tobacco chloroplasts. Mutational analysis of substrate mRNAs derived from tobacco chloroplast *psbL* and *ndhB* mRNAs confirmed the participation of *cis*-acting elements that had previously been identified *in vivo*. Competition analysis revealed the existence of site-specific *trans*-acting factors interacting with the corresponding upstream *cis*-elements. A chloroplast protein of 25 kDa was found to be specifically associated with the *cis*-element involved in *psbL* mRNA editing. Immunological analyses revealed that an additional factor, the chloroplast RNA-binding protein cp31, is also required for RNA editing at multiple sites. This combination of site-specific and common RNA-binding proteins recognizes editing sites in chloroplasts.

Keywords: chloroplast/*in vitro* system/RNA-binding protein/RNA editing/*trans*-acting factor

Introduction

Chloroplasts possess their own genome and a unique gene expression system that is regulated at the levels of transcription, RNA processing and translation, during development and in response to environmental cues (reviewed in Rochaix, 1992; Gruissem and Tonkyn, 1993; Mullet, 1993; Mayfield *et al*., 1996; Sugita and Sugiura, 1996). In chloroplasts of higher plants, many genes are initially transcribed as polycistronic precursors, which are subsequently processed into complex sets of overlapping transcripts including monocistronic mRNAs. During this process, some of the transcripts are also edited and/or spliced (Tanaka *et al*., 1987; Barkan, 1988; Westhoff and Herrmann, 1988; Neuhaus and Link, 1990; Sexton *et al*., 1990; Hird *et al*., 1991; Hoch *et al*., 1991). These RNA processing steps have been recognized as important regulatory steps in chloroplast gene expression (Deng and Gruissem, 1988; Barkan, 1989; Herrmann *et al*., 1992; Rochaix, 1992; Sugiura, 1992; Gruissem and Tonkyn, 1993; Mayfield *et al*., 1996; Sugita and Sugiura, 1996).

RNA editing is a process that alters nucleotide sequences of primary transcripts. It has been detected in divergent organisms including trypanosomes, slime mold, *Acanthamoeba*, mammals, viruses and land plants in which distinct editing mechanisms apparently operate (Benne, 1996; Simpson and Emeson, 1996; Smith *et al*., 1997). In land plants of all major lineages, RNA editing has been found both in chloroplast and mitochondrial transcripts. The major form of organellar editing involves C→U conversion, although rare U→C changes have also been detected (Covello and Gray, 1989; Gualberto *et al*., 1989; Hiesel *et al*., 1989, 1994; Hoch *et al*., 1991; Bonnard *et al*., 1992; Hanson *et al*., 1996; Maier *et al*., 1996; Yoshinaga *et al*., 1996, 1997; Freyer *et al*., 1997; Sugiura *et al*., 1998; Bock, 2000). Editing in plant organelles usually restores conserved amino acids, suggesting that it is an obligatory step in the biosynthesis of functional gene products (e.g. Bock *et al*., 1994; Maier *et al*., 1996). In chloroplasts, some transcripts undergo partial editing. In these cases, the extent of editing depends on environmental and/or developmental conditions, suggesting that editing plays a regulatory role in gene expression (Bock *et al*., 1993; Hirose *et al*., 1994, 1996, 1998; Hirose and Sugiura, 1997; Karcher and Bock, 1998). Although in land plants the chloroplast genome (~150 genes) contains more genes than its mitochondrial counterpart (60–90 genes), the total number of edited residues is much smaller: ~30 sites in chloroplasts (Maier *et al*., 1995; Wakasugi *et al*., 1996; Hirose *et al*., 1999) versus >400 sites in mitochondria (Giege and Brennicke, 1999). Using transgenic approaches in tobacco chloroplasts, the only available transformation system among plant organelles (Svab and Maliga, 1993), *cis*-acting elements important for the recognition of editing sites have been identified in *psbL* and *ndhB* mRNAs (Chaudhuri *et al*, 1995; Bock *et al*., 1996, 1997; Chaudhuri and Maliga, 1996, 1997; Herman and Bock, 1999). The involvement of extraplastidic site-specific *trans*-acting factors has also been suggested (Chaudhuri *et al*., 1995; Bock and Koop, 1997). Editing of the *psbL* mRNA *in vivo* creates the AUG initiation codon from ACG, resulting in the production of functional mRNA (Kudla *et al*., 1992). The signal directing the editing of this site is located within a 22-nucleotide-long sequence spanning the editing site (16 nt upstream and 6 nt downstream) (Chaudhuri and Maliga, 1996). In addition, an essential upstream sequence element and nucleotides critical for editing of the tobacco *ndhB* mRNA have also been identified (Bock *et al*., 1996, 1997). Recently, it has been suggested that the spacing between the upstream element and the *ndhB* editing site is crucial for substrate recognition (Hermann and Bock, 1999).

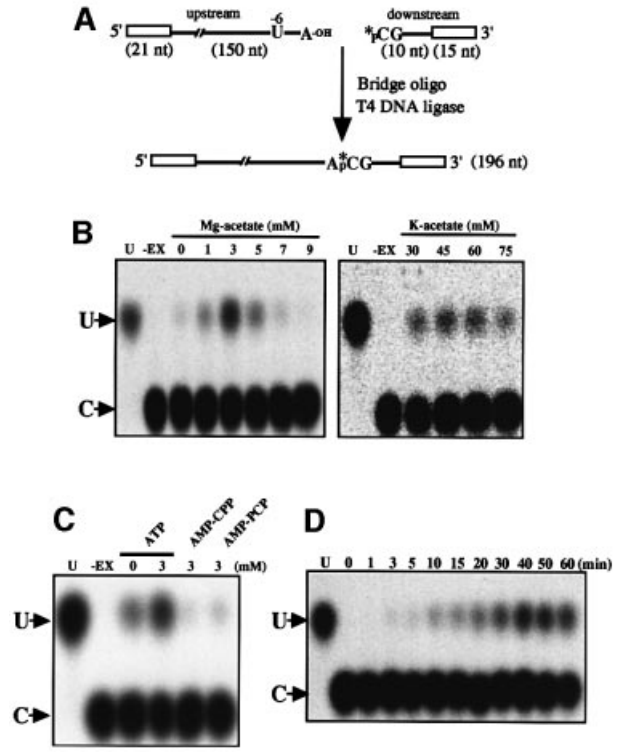

Fig. 1. *In vitro* RNA editing system. (**A**) Synthesis of the *psbL* mRNA substrate labeled at the editing site. The upstream RNA (150 nt preceding the editing site with a 5′ extension of 21 nt sequence from pBluescript II) and the downstream RNA (10 nt downstream from the editing site with the 3′ extension of a 15 nt sequence from the KS primer) are ligated with T4 DNA ligase in the presence of a bridge DNA oligonucleotide. Extensions are represented by rectangular boxes. (**B**) $[Mg^+]$ and $[K^+]$ dependencies of the *in vitro* editing reaction of *psbL* mRNA. U, marker pU; –Ex, no chloroplast extract. pU migrates slower than pC as indicated by arrows. (**C**) Effect of ATP and its analogs (AMP-PCP and AMP-CPP) on the *in vitro* editing reaction of *psbL* mRNA. (**D**) Kinetic analysis of the *in vitro* editing reaction of *psbL* mRNA.

Here, we report the development of an accurate and efficient *in vitro* RNA editing system from isolated tobacco chloroplasts. Using this system, we first confirmed that the *cis*-acting elements, which were identified by *in vivo* analysis, are important for the editing of *psbL* and *ndhB* mRNAs. The involvement of site-specific *trans*-acting factors for each edited mRNA was determined by competition analysis. UV cross-linking experiments and immunological analysis suggested that several RNA-binding proteins, but not additional RNA factors, are involved in editing. A model for the molecular mechanism of RNA editing in chloroplasts is proposed based on our results.

Results

Development of an in vitro RNA editing system from tobacco chloroplasts

In order to dissect the biochemical mechanisms of RNA editing, we have developed an *in vitro* system supporting accurate editing from tobacco chloroplasts using the *psbL* mRNA as a model substrate. Site-specific labeling of the mRNA at the edited site led to the detection of the edited products. The upstream and downstream parts of the mRNA (with respect to the C residue to be edited) were synthesized separately (Figure 1A). The 5′ end of the downstream sequence, which constitutes the editing site, was labeled with ^{32}P using polynucleotide kinase, and ligated to the upstream part with T4 DNA ligase in the presence of a complementary bridge DNA oligonucleotide (Moore and Sharp, 1992; Yang *et al*., 1995). The resulting *psbL* mRNA substrate was incubated with chloroplast S60 extract prepared as described below. After incubation at 28°C for the indicated times, RNA was isolated, digested with nuclease P1 and separated by cellulose thin layer chromatography (TLC) (Figure 1D).

Intact chloroplasts were prepared from tobacco green leaves according to the procedure used previously to prepare an *in vitro* translation system (Hirose and Sugiura, 1996). The isolated chloroplasts were lysed with 0.2% Triton X-100 in the presence of 2 M KCl, followed by centrifugation at 60 000 *g* for 2 h. The resulting S60 fractions were extensively dialyzed. The TLC analysis (Figure 1B) clearly demonstrates that a fraction of the labeled C is converted to U after incubation with the chloroplast extract. The editing activity is influenced by the concentrations of magnesium (optimal at 3 mM) and potassium (optimal at 45 mM) (Figure 1B). The addition of ATP at 3 mM strikingly enhanced the editing activity, and non-hydrolyzable ATP analogs (AMP-CPP and AMP-PCP) did not substitute for ATP, suggesting that hydrolysis of ATP is required for the efficient *in vitro* editing reaction (Figure 1C). The C→U change proceeded linearly up to 40 min and then reached a plateau (Figure 1D). These results indicate that the α-phosphate group of the edited residue is retained during the editing reaction, suggesting that the C→U conversion in chloroplasts occurs by cytidine deamination and not by nucleotide substitution as suggested for plant mitochondria (Rajasekhar and Mulligan, 1993; Yu and Schuster, 1995).

The substrate mRNA possesses 5′ and 3′ extensions comprised of vector sequences, 22 and 15 nt, respectively (see Figure 1A). After the editing reaction, cDNA amplified from the isolated substrate mRNA by RT–PCR using primers complementary to the vector extensions (to avoid background from endogenous *psbL* mRNAs) yielded a single band. Sequence analysis of the cloned cDNAs revealed that C→U conversion occurred exclusively at the authentic editing site in eight cDNAs out of the 64 clones examined (56 cDNAs were not edited) (data not shown). These results confirm that the *in vitro* system supports accurate RNA editing of the *psbL* mRNA.

Cis-acting elements for the editing of psbL and ndhB mRNAs

Cis-acting elements involved in editing have previously been identified using *in vivo* transplastome analysis of tobacco *psbL* and *ndhB* mRNAs (sites IV and V) (Bock *et al*., 1996; Chaudhuri and Maliga, 1996). In order to confirm whether our *in vitro* editing system depends on these *cis*-acting elements, mutational analysis of tobacco *psbL* and *ndhB* (site IV) mRNAs was performed (Figure 2A). Substitution of either the 16 nt element, the entire upstream or the 9 nt downstream sequence by a vector sequence abolished editing, indicating that both

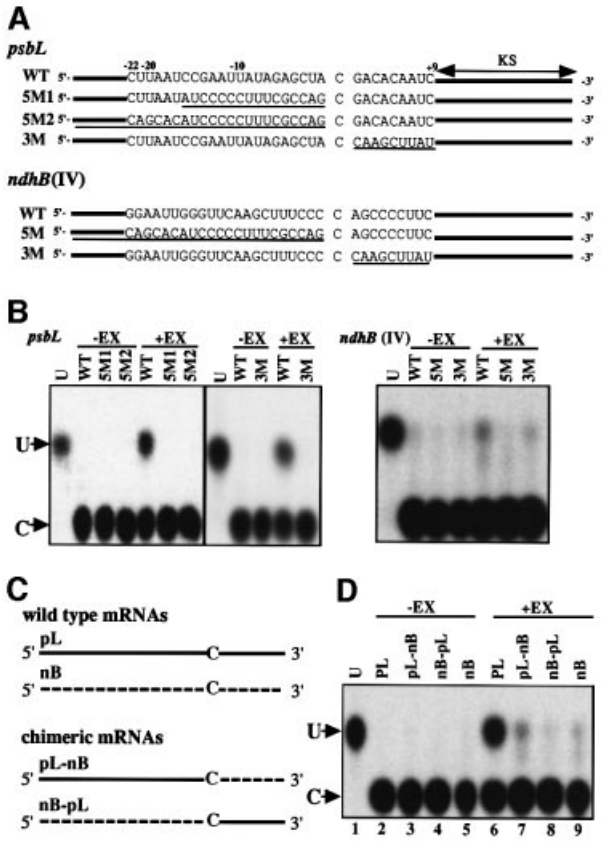

Fig. 2. *In vitro* editing using *psbL* and *ndhB* mRNAs with substitution mutations. (**A**) Sequences of wild-type (WT) and mutant (5M and 3M) *psbL* and *ndhB* mRNAs. Sequences of 22 nt upstream and 9 nt downstream of the respective editing sites are shown. Substitutions by a vector sequence are underlined. KS represents the sequence of the KS primer annealing region. WT sequences were from Shinozaki *et al.* (1986). (**B**) *In vitro* editing activity of mutant *psbL* and *ndhB* mRNAs. U, marker pU; +EX and –EX, with and without chloroplast extracts, respectively. (**C**) Schematic representation of chimeric mRNAs. (**D**) *In vitro* editing activity of chimeric mRNAs.

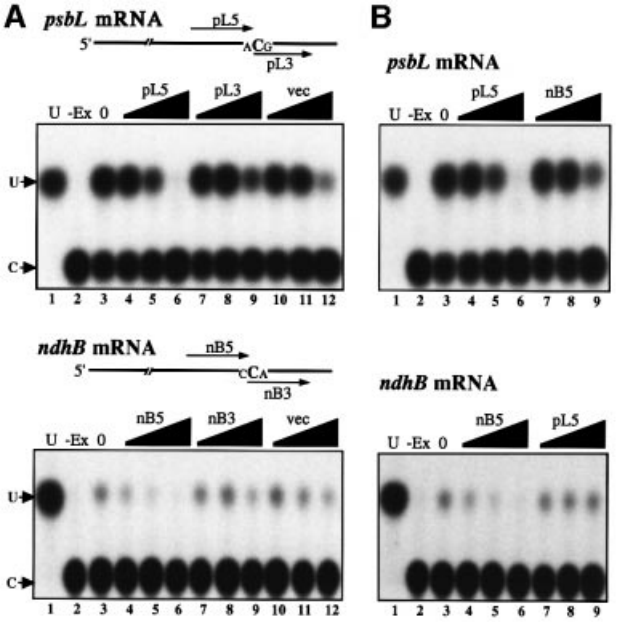

Fig. 3. Competition analysis of *in vitro* RNA editing. (**A**) Increasing amounts of upstream (pL5 and nB5), downstream (pL3 and nB3) and vector (vec) oligoribonucleotides were added to *in vitro* editing reactions with *psbL* and *ndhB* mRNAs. pL5, pL3 and vec oligos of 1 µmol (lanes 4, 7 and 10), 10 µmol (lanes 5, 8 and 11) and 100 µmol (lanes 6, 9 and 12) were added. nB5, nB3 and vec oligos of 0.25 µmol (lanes 4, 7 and 10), 2.5 µmol (lanes 5, 8 and 11) and 25 µmol (lanes 6, 9 and 12) were added. U, authentic pU; –Ex, no chloroplast extract; 0, no competitor. (**B**) Analysis with heterologous competitors. nB5 (1, 10 and 100 µmol, lanes 7, 8 and 9, respectively) was added for *psbL* mRNA. pL5 (0.25, 2.5 and 25 µmol, lanes 7, 8 and 9, respectively) was used for *ndhB* mRNA.

upstream and downstream regions are essential for editing of *psbL* mRNAs (Figure 2A and B). This result confirmed that obtained from the *in vivo* analysis using transplastomic tobacco plants (Chaudhuri and Maliga, 1996). A similar analysis of the *ndhB* mRNA showed that replacement of only the upstream, but not the downstream region inhibited editing completely. This result indicates the absolute requirement of the upstream, but not the downstream sequence for editing (Figure 2A and B), again confirming the *in vivo* result (Bock *et al.*, 1996).

Next, to examine whether the *cis*-element is effective for another editing site, chimeric substrates between *psbL* and *ndhB* mRNAs were constructed: the pL-nB substrate contained the *psbL* upstream and the *ndhB* downstream sequences, and vice versa for nB-pL (Figure 2C). As shown in Figure 2D, the chimeric pL-nB mRNA was edited *in vitro* at a low level (~10% of the wild-type *psbL* mRNA) (lane 7), whereas no editing product was observed for the nB-pL mRNA (lane 8). These results indicate that authentic combinations of the upstream and downstream sequences are required for efficient editing. It should be noted that the effects of the downstream sequence differ highly from those of an unrelated vector sequence (see Figure 2B, lane 3M).

Site-specific trans-acting factors are involved in RNA editing in chloroplasts

To examine the involvement of *trans*-acting factors in editing, competition analyses using 25 nt oligoribonucleotides corresponding to upstream and downstream regions of the editing sites in *psbL* or *ndhB* (site IV) mRNAs were carried out. As shown in Figure 3A, the editing of *psbL* and *ndhB* mRNAs was arrested by the addition of excess amounts of the upstream competitors pL5 and nB5, respectively (lanes 4–6), but not by the downstream competitors pL3 and nB3 (lanes 7–9) nor by an unrelated oligonucleotide derived from a vector sequence (lanes 10–12), although non-specific inhibition was observed in a 2000-fold excess of these competitors (lanes 9 and 12). These results strongly suggest the existence of a *trans*-acting factor(s) specifically interacting with the upstream region of each mRNA. The competitor RNAs were then exchanged. The editing of *psbL* mRNA was not arrested by an excess of nB5, the competitor for *ndhB* mRNA; similarly, the editing of *ndhB* was not inhibited by pL5 (Figure 3B, lanes 7–9), suggesting that the *trans*-acting factors are site specific. This result is consistent with observations using transplastomic tobacco plants where the introduction of additional copies of *psbL* and *ndhD* editing sites specifically reduced the editing efficiency

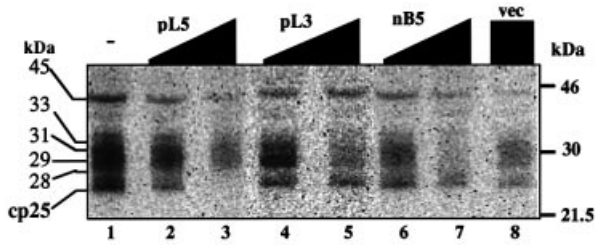

Fig. 4. Detection of tobacco chloroplast proteins bound to the upstream region of *psbL* mRNA by UV cross-linking. The *psbL* mRNA with ^{32}P-labeled U at position –6 was synthesized as in Figure 1A. The mRNA was incubated for 15 min at 28°C in the editing reaction mixture and then UV-irradiated (254 nm). The isolated RNA was digested with RNase A followed by separation of the cross-linked proteins by SDS–PAGE (lane 1). Increasing amounts (10 µmol, lanes 2, 4 and 6; 100 µmol, lanes 3, 5, 7 and 8) of competitor RNAs (see Figure 3A) were added. Protein size markers are shown at the right (Rainbow, Amersham).

of the corresponding sites in the endogenes as well as in transgenic mRNAs (Chaudhuri *et al*., 1995; Chaudhuri and Maliga, 1996).

Involvement of a 25 kDa protein in editing of psbL mRNA

The most intriguing question about *trans*-acting factors for RNA editing in chloroplasts is whether they contain an RNA component(s). To answer this question, the chloroplast extract was pre-treated with micrococcal nuclease. The editing activity did not decrease even though a 10 times higher concentration of the nuclease was applied than that used for the preparation of chloroplast *in vitro* translation systems (Hirose and Sugiura, 1996) (data not shown). Furthermore, attempts to detect RNA molecules interacting with *psbL* mRNAs by cross-linking in the presence of AMT (4′-aminomethyl-4,5′,8-trimethylpsoralen), which forms covalent adducts after irradiation with long-wavelength (365 nm) UV light, yielded negative results (data not shown). Although these experiments are indirect and preliminary, the *trans*-factor is likely to be a protein rather than an RNA.

To detect proteins interacting with the upstream *cis*-element, UV cross-linking (254 nm) experiments were carried out using the *psbL* mRNA substrate labeled at the –6 residue with respect to the C to be edited (see Figure 1A). As shown in Figure 4, at least five chloroplast proteins ranging in size from 25 to 45 kDa were detected. Competition analysis revealed that binding of the 25 kDa protein (p25) to *psbL* mRNA was arrested by an excess amount of pL5 (lane 3), but not by pL3, nB5 or vec (lanes 5, 7 and 8). Similar experiments using the *ndhB* mRNA in which the –6 position was specifically labeled were carried out; however, binding of neither p25 nor any additional protein other than proteins ranging in size from 28 to 45 kDa was observed (data not shown). The characteristics of p25 binding to *psbL* mRNA corresponds to that of editing activity (see Figure 3), strongly suggesting that p25 is a *trans*-acting factor specific for the editing of *psbL* mRNA. On the other hand, the failure to detect any specific protein binding to the *ndhB* mRNA might be due to a lower amount of the factor in the chloroplast extract or co-migration in the gel with the 28 to 33 kDa bands.

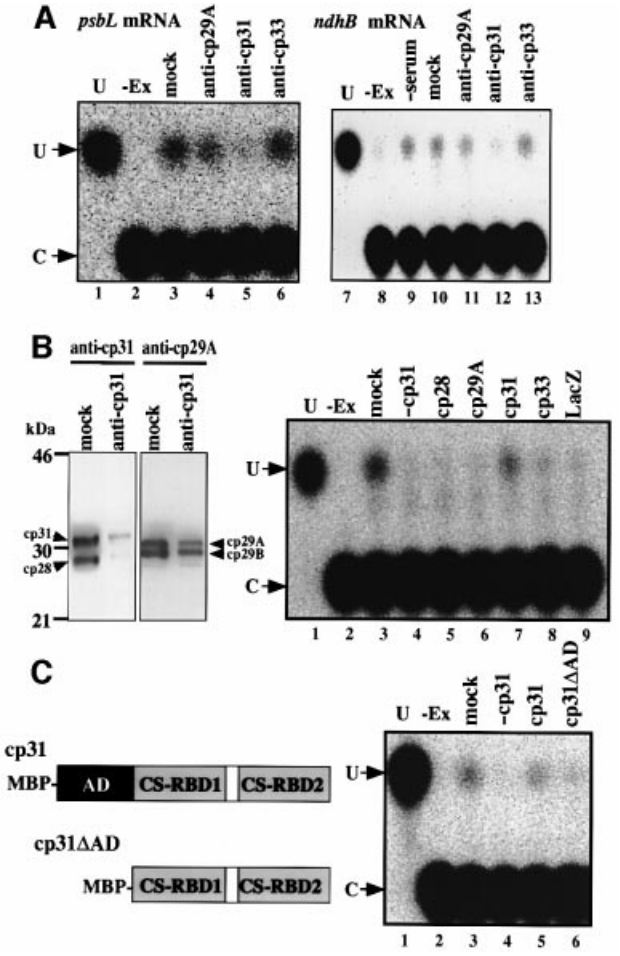

Fig. 5. Involvement of a chloroplast ribonucleoprotein, cp31, in RNA editing. (**A**) Effect of antisera against tobacco chloroplast RNPs on the editing of *psbL* and *ndhB* mRNAs *in vitro*. (**B**) Immunodepletion of cp31 from the chloroplast extract (left panel) and the effect of recombinant RNPs (0.2 µg each) on editing of *psbL* mRNA using the cp31-depleted extract (right panel). Western blot patterns of the chloroplast extract with treatment with anti-cp31 (anti-cp31) or with pre-immune serum (mock). Detection was with anti-cp31 (left) and anti-cp29A (right) (note that anti-cp31 reacts with cp31 and 28, and anti-cp29A with cp29A and -B). (**C**) Effect of the AD of cp31 on editing of *psbL* mRNA. Structures of the recombinant cp31 and that lacking the AD (cp31ΔAD). The N-terminal extension is maltose-binding protein (MBP). cp31 and cp31ΔAD (0.2 µg each) were added to the cp31-depleted extract (lanes 5 and 6, respectively). U, authentic pU; –Ex, no chloroplast extract; mock, extracts treated with pre-immune serum; –cp31, extract treated with anti-cp31; *lacZ*, recombinant MBP-*lacZ* protein as a control.

A chloroplast RNA-binding protein, cp31, is a common factor for editing of psbL and ndhB mRNAs

Based on their sizes, the additional cross-linked proteins of 28–33 kDa are considered to be chloroplast ribonucleoproteins (cpRNPs: cp28, cp29A, cp29B, cp31 and cp33) previously isolated and characterized by our laboratory (Li and Sugiura, 1990; Ye *et al*., 1991). Each cpRNP is an abundant stromal protein that possesses two consensus-type RNA-binding domains (CS-RBD) and an N-terminal acidic domain (AD).

These cpRNPs are associated with various chloroplast RNA species including mRNAs, pre-tRNAs and pre-rRNAs (Nakamura *et al*., 1999), suggesting that they are involved in RNA processing events and/or RNA stability/storage.

To verify the involvement of these cpRNPs in editing, we investigated the effect of antibodies against each cpRNP on the *in vitro* editing reactions. As shown in Figure 5A, the editing of both *psbL* and *ndhB* mRNAs was inhibited by the addition of anti-cp31, which can recognize cp28 as well as cp31 (lanes 5 and 12), while the addition of antibodies against other cpRNPs (anti-cp29A recognizes cp29A and B, while anti-cp33 recognizes only cp33) did not affect the editing activity (lanes 4, 6, 11 and 13). We then depleted cp31 from the chloroplast extract by immunoprecipitation with anti-cp31. As shown in Figure 5B (left panel), practically no cp31 (and no cp28) was present in the extract, whereas cp29A and B were still detected. Immunodepletion of cp31 from the editing extract resulted in the inhibition of *psbL* mRNA editing (Figure 5B, right panel, lane 4), and the addition of recombinant cp31 (0.2 μg), but not recombinant cp28, back into the cp31-depleted extract restored the editing activity (lane 7). These results indicate that cp31 is an additional essential factor involved in the editing of *psbL* and *ndhB* mRNAs, suggesting that cp31 is a common factor for RNA editing in tobacco chloroplasts. Furthermore, the addition of excess amounts (2 μg) of recombinant cp31 into the cp31-depleted extract and also into the untreated extract partially inhibited the editing of *psbL* mRNA (data not shown), suggesting that an appropriate concentration of cp31 is important for the editing activity.

CS-RBD1 and CS-RBD2 in cp28 and cp31 are highly conserved (85–88% identity), but their ADs are not (Li and Sugiura, 1990). Among all five cpRNPs, cp31 has the longest AD (64 amino acids). To investigate the function of the cp31 AD, recombinant cp31 lacking AD (cp31ΔAD) fused to maltose-binding protein (MBP) was prepared (Figure 5C). When cp31ΔAD was added to the cp31-depleted extract, editing was hardly detected (Figure 5C, right panel, lane 6), indicating that the AD is necessary for the function of cp31 in editing. Therefore, the AD may assist the assembly of the editing machinery.

Discussion

Development of a chloroplast in vitro RNA editing system

The RNA editing process of C→U conversion is found both in chloroplasts and mitochondria of most land plants. However, the molecular mechanism of editing in plant organella is not well understood. Two intriguing questions for the editing mechanism can be raised. (i) How are editing sites specifically recognized? (ii) What is the catalytic mechanism? In tobacco chloroplast transcripts, a total of 31 editing sites have been identified so far, and sequences surrounding these editing sites lack similarity except for the bias towards a pyrimidine residue at position –1 and an adenine residue at position +1 (Hirose *et al*., 1999). Therefore, it is possible that each editing site is recognized by site-specific factors. *In vivo* observations using transplatomic tobacco plants have already suggested the involvement of site-specific factors (Chaudhuri *et al*., 1995; Bock and Koop, 1997). However, chloroplast transformation techniques have their own limitations for biochemical analysis of editing processes. In plant mitochondria, an *in vitro* RNA editing system was described from wheat mitochondria (Araya *et al*., 1992). However, no further analysis has been reported.

In order to dissect the molecular mechanism of RNA editing in more detail, we have succeeded in developing a genuine *in vitro* system supporting accurate RNA editing from isolated tobacco chloroplasts. We utilized tobacco leaves at the defined stage (5–10 cm, grown in a growth chamber) previously selected for the preparation of our chloroplast *in vitro* translation system (Hirose and Sugiura, 1996). To assay editing activity, RNA substrates specifically labeled with ^{32}P at the 5′ C, which undergoes editing, were prepared, leading to sensitive detection of the editing product without background. Detection of [5′-^{32}P]UMP as the edited product strongly suggests that the catalytic mechanism of RNA editing in chloroplasts involves cytidine deamination as in the case of plant mitochondria (Rajasekhar and Mulligan, 1993; Yu and Schuster, 1995). The accuracy of this system was confirmed by sequencing of cDNA clones derived from the RNA substrate after an *in vitro* editing reaction. Among various variables for the preparation of chloroplast extracts and for the *in vitro* reactions, the magnesium concentration (sharp optimum at 3 mM) is one of the most important. Hydrolysis of ATP is likely to be required for efficient editing of chloroplast mRNAs (see Figure 1C). ATP is not required for the mechanism of catalysis by cytidine deaminase (Frick *et al*., 1989). The requirement for ATP has not been reported for the process of C→U editing of *apoB* mRNAs in mammals (Driscoll *et al*., 1989). It is therefore suggested that, in chloroplasts, ATP is utilized for editing site recognition or assembly of editing complexes rather than at the catalytic step.

Involvement of two distinct RNA-binding proteins in psbL mRNA editing

Our *in vitro* analysis using two different RNA editing sites revealed that site-specific *trans*-acting factors recognize upstream *cis*-acting elements of respective editing sites. In trypanosome mitochondria, editing sites are determined by sets of small RNAs, termed guide RNA (Blum *et al*., 1990). More than 100 small nucleolar RNAs (snoRNAs) interact with pre-rRNAs to define the sites of sugar methylation and pseudouridylation in the nucleolus of eukaryotic cells (Bachellerie *et al*., 1995; Tollervey, 1996; Smith and Steitz, 1997). Thus, *trans*-acting RNA molecules are widely utilized as guide RNAs for post-transcriptional modification of specific sites in many precursor transcripts from various organisms. However, plastid transformation tests failed to detect guide RNAs for *psbL* mRNA editing (Bock and Maliga, 1995). Our *in vitro* analyses using micrococcal nuclease treatment and psolaren cross-linking also failed to detect RNA factors involved in the RNA editing reaction. Instead, an RNA-binding protein of 25 kDa (cp25) appears to bind specifically to the *cis*-acting element of *psbL* mRNA. This result strongly suggests that a chloroplast-specific protein, but not an RNA factor, recognizes the *cis*-acting

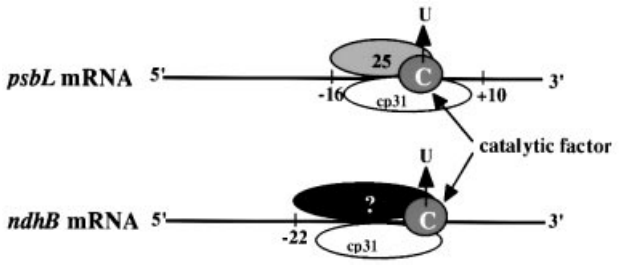

Fig. 6. Model for the mechanism of RNA editing in chloroplasts. A site-specific *trans*-acting factor (p25 for tobacco *psbL* mRNA) binds to the upstream *cis*-acting element of an editing site. One of the abundant chloroplast RNPs (cp31 in tobacco), probably a common factor, also binds close to every editing site. Complexes including these proteins recruit the catalytic factor of C→U conversion to the editing sites. Numbers represent *cis*-element positions defined by transplastomic experiments (Bock *et al*., 1996; Chaudhuri and Maliga, 1996).

element and the C that undergoes conversion to a U residue.

cp31 belongs to a family of abundant chloroplast RNA-binding proteins with an N-terminal AD and two CS-RBDs (Li and Sugiura, 1990). It has been suggested to be involved in RNA processing, RNA storage and/or translation in chloroplasts (Nakamura *et al*., 1999). A spinach homolog of tobacco cp28 called 28RNP has been shown to be necessary for accurate 3′ end formation of chloroplast mRNAs *in vitro* (Schuster and Gruissem, 1991). Depletion of all five cpRNPs (cp28, 29A, 29B, 31 and 33) from tobacco chloroplast extracts did not affect the rate of 3′ processing of *petD* mRNA (Nakamura *et al*., 1999), whereas the addition of anti-cp31 (recognizing both cp28 and 31) abolished *in vitro* editing of both *psbL* and *ndhB* mRNAs (see Figure 5A), suggesting the involvement of either cp28 or cp31 in RNA editing. A set of experiments with depletion or addition of cp28 and cp31 to our extracts revealed that cp31, but not cp28, is required for editing. Additional experiments using the mutant cp31 lacking the AD have revealed that this domain is important for the function of cp31 in RNA editing. cp31 is a highly abundant stromal protein whose abundance per chloroplast is estimated to be higher than that of chloroplast ribosomes (T.Nakamura, M.Sugiura and M.Sugita, in preparation). On the other hand, the chloroplast transcripts possessing RNA editing sites are limited among total chloroplast RNAs, suggesting that cp31 is a multifunctional protein involved in several other post-transcriptional events as well as RNA editing. In mammalian nuclei, a set of heterogeneous nuclear ribonucleoproteins (hnRNPs) interacts with newly transcribed pre-mRNAs (Burd and Dreyfuss, 1994), and each hnRNP governs multiple pre-mRNA processing steps including RNA splicing, RNA stability and mRNA export to the cytoplasm. cp31 is thought to act as an hnRNP-like protein in chloroplasts for multiple post-transcriptional processes, including RNA editing. It is also possible that the other chloroplast RNA-binding proteins (cp28, 29A, 29B and/or 33) are involved in editing of mRNAs other than *psbL* and *ndhB* (site IV). Alternatively, cp31 may play a more indirect role in editing; namely, it may facilitate efficient editing by mediating the accessibility of editing sites to the editing machinery instead of being a part of the editing complex.

Mechanism of RNA editing in chloroplasts

Based on our *in vitro* analyses, we propose a model for the mechanism of RNA editing in chloroplasts (Figure 6). A site-specific *trans*-acting factor interacts with the upstream *cis*-acting element and determines the cytidine residue to be edited. Tobacco p25 is a strong candidate for the site-specific *trans*-acting factor for editing of tobacco *psbL* mRNA. Another factor involved in editing in tobacco is an abundant RNA-binding protein, cp31, which is likely to act as a common factor for the editing of multiple sites. The catalytic factor, probably RNA cytidine deaminase, is then recruited to the editing site.

In the case of *apoB* mRNA editing in mammalian nuclei, the catalytic factor apobec-1 is an RNA cytidine deaminase (Navaratnam *et al*., 1993, 1995; Teng *et al*., 1993), whose recruitment to the editing site requires a downstream 'mooring sequence' and a factor(s) interacting with this element (Mehta *et al*., 1996; Schock *et al*., 1996). Recently, the 'mooring sequence'-binding factor has been purified and its cDNA cloned, leading to a model for the editing complex in which this factor (termed ACF) binds to the mooring sequence and docks apobec-1 to deaminate its target cytidine (Mehta and Driscoll, 1998; Mehta *et al*., 2000). In chloroplasts, the upstream sequence may correspond to the 'mooring sequence' of the *apoB* editing system. cp31 is unlikely to have RNA cytidine deaminase activity due to the lack of any known deaminase motif (Li and Sugiura, 1990), suggesting that an additional factor is required to catalyze cytidine deamination. Several genes for proteins containing the deaminase motif have been isolated from *Arabidopsis*; however, none of their products has been proven to be imported to chloroplasts (Faivre-Nitschke *et al*., 1999). Searching for additional genes encoding cytidine deaminase-like proteins in the *Arabidopsis* genome database could be one effective way of finding the catalytic factor.

Our *in vitro* experiments raised an interesting possibility that each editing site is recognized by a unique *trans*-acting factor. Since we previously detected at least 31 editing sites in tobacco chloroplasts, ~31 different *trans*-factors would be needed, which are likely to be encoded in the nuclear genome and transported into chloroplasts. Alternatively, different combinations of abundant chloroplast RNA-binding proteins (e.g. cp31) and a limited number of *trans*-factors (e.g. p25) may affect the recognition of editing sites. Further *in vitro* analyses using other chloroplast mRNAs will provide the answers to the above questions. C→U RNA editing is also observed in plant mitochondria, where editing is 10 times more frequent. It has been reported that a mitochondrial editing site is not recognized by the chloroplast editing machinery (Sutton *et al*., 1995). However, the bias of the nucleotides at position –1 and position +1 at the mitochondrial editing sites is similar to that in chloro-plasts, suggesting that the mechanism of editing is also similar. It will be interesting to determine how and to what extent, if any, editing machineries in both plant organelles overlap.

Materials and methods

Preparation of substrate mRNAs

The upstream RNA was synthesized using the T3 MEGASCRIPT RNA synthesis kit (Ambion), and the tobacco *psbL* clone containing the region

from –150 to –1 and the 5′ extension of a 21 nt sequence from pBluescript II, which had been linearized with *EcoT22*I and blunted (see Figure 1A). The downstream RNA including the C to be edited and the 3′ extension of a 15 nt sequence from the KS primer was chemically synthesized by TaKaRa. The downstream RNA (300 pmol) was labeled with ^{32}P at the 5′ end with T4 polynucleotide kinase and purified by passage through a Sephadex G25 spin column (Amersham Pharmacia Biotech). The labeled downstream RNA was mixed with the upstream RNA (100 pmol) and the bridge DNA oligonucleotide (200 pmol) (5′-CGGTATCGGATTGTGTCGTAGCTCTATAATTCGGATTAAG-3′), and heated at 94°C for 3 min followed by cooling to room temperature for 3 h. Ligation was carried out by adding 1.4 U/µl T4 DNA ligase (TaKaRa) and incubating at 25°C overnight. The ligated RNA was purified by PAGE. The *ndhB* (site IV) mRNA substrate was prepared as above and includes 156 nt upstream and 10 nt downstream sequences with respect to the editing site. Chimeric mRNA substrates were prepared as above (Figure 2C, left panel).

Preparation of chloroplast S60 fractions and in vitro RNA editing reaction

Tobacco (*Nicotiana tabacum* var. Blight Yellow 4) was grown in a growth chamber (28°C, 16 h light and 8 h dark) for 4 weeks. Intact chloroplasts (~500 µl packed volume) were prepared from ~150 g of tobacco green leaves of 5–10 cm in length (stage III; Hirose and Sugiura, 1996), disrupted and extracted with 400 µl of extraction buffer [30 mM HEPES–KOH pH 7.7, 10 mM magnesium acetate, 2 M KCl and 2 mM dithiothreitol (DTT)] containing 0.2% Triton X-100, and centrifuged at 6000 *g* for 10 min. The transparent crude supernatant was subjected to ultra-centrifugation at 60 000 *g* for 2 h. The supernatant (S60 fraction) was recovered and dialyzed against dialysis buffer containing 30 mM HEPES–KOH pH 7.7, 3 mM magnesium acetate, 45 mM potassium acetate, 30 mM ammonium acetate and 10% glycerol for 12 h. All steps were carried out at 0–2°C. Reaction mixtures (25 µl) consisted of 30 mM HEPES–KOH pH 7.7, 3 mM magnesium acetate, 45 mM potassium acetate, 30 mM ammonium acetate, 3 mM ATP, 2 mM DTT, 1% polyethyleneglycol 6000, 5% glycerol, 230 U of RNase inhibitor (TaKaRa), 1× Proteinase inhibitor mixture (Complete™, Boehringer Mannheim), 50 fmol of [^{32}P]mRNA substrate and chloroplast S60 fraction (250 µg of protein). The *in vitro* editing reaction was carried out at 28°C for 40 min or as indicated. The substrate mRNA was extracted with phenol–chloroform and precipitated with ethanol. The RNA was digested into 5′ mononucleotides by 1 µg of nuclease P1 in the presence of 50 mM ammonium acetate pH 4.8 at 37°C for 3 h. The resultant mononucleotides were separated on cellulose TLC plates using isopropanol:HCl:water (25:24:1) for the mobile phase. The separated [^{32}P]mononucleotides were visualized and quantified by a Bioimaging Analyzer BAS2000 (Fuji Photo Film Co). Competitor RNA oligonucleotides of 25 nt were chemically synthesized by TaKaRa (see Figure 3A).

UV cross-linking experiments

The *psbL* mRNA substrate specifically labeled with ^{32}P at U position –6 with respect to the editing site was synthesized as above (see Figure 1A). Ten femtomoles of ^{32}P-labeled mRNA were incubated under *in vitro* editing conditions at 28°C for 15 min. The reaction mixture was irradiated with UV light (254 nm, 1.8 J/cm^2) using Funacrosslinker (Funakoshi Co.) followed by digestion of the RNA with RNase A at 37°C for 15 min. The protein samples were precipitated with 50% acetone and dissolved in SDS–PAGE loading buffer followed by separation on 15% polyacrylamide gels containing 0.1% SDS. The separated proteins were visualized as described above.

Immunodepletion analysis

Recombinant cpRNPs with MBP and their antibodies were prepared and provided by Dr Masaru Ohta (Nakamura *et al*., 1999). Recombinant cp31 lacking the AD was prepared by the same procedure. Immunodepletion of cp31 (and cp28) was carried out essentially as described (Nakamura *et al*., 1999). The chloroplast extract (~100 µg protein) was mixed with anti-cp31–protein A–Sepharose beads (10 mg) for 1 h, followed by dialysis against 2 l of the dialysis buffer used for S60 preparation. Depletion of cp31 was confirmed by western blot analysis using anti-cp31 and anti-cp29A (control). The recombinant cpRNPs were dialyzed against the buffer used for S60 preparation, and 0.2 µg of each cpRNP were added to the *in vitro* editing reaction.

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