Recognition of RNA Editing Sites Is Directed by Unique Proteins in Chloroplasts: Biochemical Identification of *cis*-Acting Elements and *trans*-Acting Factors Involved in RNA Editing in Tobacco and Pea Chloroplasts

Tetsuya Miyamoto,¹ Junichi Obokata,¹ and Masahiro Sugiura^{2*}

Center for Gene Research, Nagoya University, Nagoya 464-8602,¹ and Graduate School of Natural Sciences, Nagoya City University, Yamanohata, Mizuho, Nagoya 467-8501,² Japan

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RNA editing in higher-plant chloroplasts involves C-to-U conversions at specific sites. Although in vivo analyses have been performed, little is known about the biochemical aspects of chloroplast editing reactions. Here we improved our original in vitro system and devised a procedure for preparing active chloroplast extracts not only from tobacco plants but also from pea plants. Using our tobacco in vitro system, *cis*-acting elements were defined for *psbE* and *petB* mRNAs. Distinct proteins were found to bind specifically to each *cis*-element, a 56-kDa protein to the *psbE* site and a 70-kDa species to the *petB* site. Pea chloroplasts lack the corresponding editing site in *psbE* since T is already present in the DNA. Parallel in vitro analyses with tobacco and pea extracts revealed that the pea plant has no editing activity for *psbE* mRNAs and lacks the 56-kDa protein, whereas *petB* mRNAs are edited and the 70-kDa protein is also present. Therefore, coevolution of an editing site and its cognate *trans*-factor was demonstrated biochemically in *psbE* mRNA editing between tobacco and pea plants.

RNA editing is a posttranscriptional process that alters nucleotide sequences of primary transcripts in a variety of organisms, including trypanosomes, mammals, and land plants (2, 12, 45, 46). In land plants, RNA editing was first reported in mitochondrial transcripts (17, 24, 27) and then in chloroplast mRNAs (32, 33). The major form of plant organellar editing involves a C-to-U conversion, although rare U-to-C changes have also been detected (9, 10, 11, 25, 36, 38, 39, 49). Most editing events restore conserved amino acids, suggesting that it is an obligatory step in the biosynthesis of functional gene products (3, 42, 60).

The occurrence of RNA editing was investigated in various species and was detected in the mitochondria and chloroplasts of all major lineages of land plants (22, 28, 37). However, editing frequencies show remarkable variation, being high in mitochondria and low in chloroplasts. The editing sites of mitochondria and chloroplasts were systematically analyzed in several species whose organellar genomes were entirely sequenced. To date, 441 editing sites have been identified in mitochondrial transcripts from *Arabidopsis* spp. (23), while the number of editing sites in chloroplast mRNAs is 27 in maize (5, 35), 26 in black pine (53), 31 in tobacco (30), and 21 in rice (16). The bryophyta (moss) is an exception, with extensive editing of C-to-U and U-to-C changes observed in the hornwort *Anthoceros formosae* (58, 59), whereas no editing has been reported in the liverwort *Marchantia polymorpha*.

Sequences surrounding the 31 editing sites identified in tobacco chloroplast transcripts were compared and found to

include no obvious consensus sequence or secondary structure (30). The situation is also similar for maize, rice, and other species. Therefore, a key question is how specific C residues are recognized precisely from all other C residues in the transcripts. Employing transgenic methodologies to tobacco chloroplasts, *cis*-acting elements have been intensively analyzed for *psbL* mRNAs (14, 15), for *ndhB* mRNAs (sites IV and V) (4, 6, 26), and for *rpoB* mRNAs (site II) (41). These studies showed that *cis*-acting elements commonly reside in upstream regions of the editing sites. The editing of *apoB* mRNAs in mammals is perhaps the best-characterized example of C-to-U editing, which produces a premature stop codon (18). In contrast with chloroplast RNA editing, the *cis*-acting element of *apoB* mRNA resides in the downstream region of the editing site (19).

Chloroplast transplastomic experiments suggested the involvement of *trans*-acting factors in editing (7, 14, 15, 40). At least some *trans*-factors appear to be site specific and of extraplastidic origin; however, the molecular entity of these factors has not been identified. Recently, an in vitro RNA editing system from tobacco chloroplasts was developed in our laboratory as a means of investigating the biochemical processes of editing reactions in chloroplasts (31). Using this system, a 25-kDa tobacco chloroplast protein (p25) was found to bind specifically to the *cis*-acting element of *psbL* mRNA. This provided evidence that RNA-binding protein, not guide RNA, is a candidate for the *trans*-acting factor that recognizes the editing site of *psbL* mRNAs.

In the present study, we report an improved method for preparing chloroplast extracts supporting accurate RNA editing reactions in vitro not only from tobacco but also from an additional plant species, pea. The availability of a pair of in vitro extracts (from tobacco and pea) has enabled us to com-

* Corresponding author. Mailing address: Graduate School of Natural Sciences, Nagoya City University, Yamanohata, Mizuho, Nagoya 467-8501, Japan. Phone: 81-52-872-6021. Fax: 81-52-872-6021. E-mail: sugiura@nsc.nagoya-cu.ac.jp.

pare the editing mechanism between the two different species. This approach revealed the correlation between editing activities and the existence of site-specific protein factors involved in the editing of chloroplast transcripts.

MATERIALS AND METHODS

Preparation of mRNA substrates. RNA substrates were prepared essentially as described previously (31). The upstream region of the tobacco *psbE* gene was amplified by PCR from plasmid pTS9 DNA (48) by using primers 5'-GCGGG GCCCTATTCATTGCGGGTTGGTTATTG-3' and 5'-GCGTCTAGATCAA AACGGCCAGTTATTAATGG-3'. The product (−128 to −1) was inserted into the *ApaI*-*XbaI* site of pBluescript II KS(+) (KS). The plasmid was linearized with *XbaI* and blunted, followed by in vitro transcription by using the T3 Megascript RNA synthesis kit (Ambion). The transcript includes the 5' extension of a 20-nucleotide (nt) sequence from KS. The downstream RNA (11 nt, beginning with the C nucleotide to be edited) and the 3' extension (a 17-nt sequence from KS) were chemically synthesized by TaKaRa. The downstream RNA (20 pmol) was labeled with ³²P at the 5' end with T4 polynucleotide kinase and purified by passage through a Sephadex G-25 spin column (Amersham Pharmacia Biotech). The labeled downstream RNA was mixed with the upstream RNA (60 pmol) and a bridge DNA oligonucleotide (40 pmol, 5'-GACGGTATCGTGTCCAAAG GATCAAACGCGCCAGTTATTA-3'). This was heated at 94°C for 3 min and then allowed to cool at room temperature for 2 h. Ligation was carried out by the addition of 3 U of T4 DNA ligase (TaKaRa)/μl, followed by incubation at 25°C overnight. The ligated mRNA was purified by polyacrylamide gel electrophoresis (PAGE). The upstream region of the tobacco *petB* gene was amplified by PCR from plasmid pTBa1 DNA (48) with primers 5'-GCGGGGCCCTTGGTCGAA TTATTGCGCGGAAG-3' and 5'-GCGCTGCAGAAAGTGCATTAACATAA ATACGG-3'. The product (−121 to −1) was inserted into KS, linearized with *PstI*, and subjected to in vitro transcription as described above. The downstream RNA of 11 nt was synthesized and labeled as described above. The upstream and downstream RNAs were ligated as described above with a bridge oligonucleotide DNA (5'-GACGGTATCGACGTATCATTGGAAGTGCATTAACATAAAA T-3'). The *psbE* and *petB* mRNAs for UV cross-linking experiments were prepared by a similar manner.

Preparation of improved tobacco chloroplast extracts. Tobacco (*Nicotiana tabacum* var. Bright Yellow 4) was grown in a growth chamber at 25°C (instead of the previous 28°C) by using a white light (fluorescent, 50 microeinsteins/m² · s) in a 16-h light and 8-h dark cycle for 6 weeks. Intact chloroplasts were prepared by the method of Bartlett et al. (1) with modifications. About 100 g of expanded leaves (5 to 10 cm in length) in 300 ml of MCB1 (0.3 M mannitol; 50 mM HEPES-NaOH, pH 8.0; 2 mM EDTA; 5 mM β-mercaptoethanol) with 0.1% bovine serum albumin and 0.6% polyvinylpyrrolidone (molecular weight, ~40,000) were disrupted by using a Polytron homogenizer (Kinematica). The extract was filtered through four layers of cotton gauze, and the filtrate was centrifuged at 1,000 × g for 10 s. The green pellet was resuspended in 6 ml of MCB1 containing 0.1% bovine serum albumin, and 3-ml portions were loaded separately onto two 10 to 80% Percoll linear gradients (40 ml each) in MCB1. After centrifugation at 6,000 × g (4°C) for 15 min, the lower green band was collected (~10 ml) and mixed with 3 to 5 volumes of MCB2 (0.32 M mannitol; 50 mM HEPES-NaOH, pH 8.0; 2 mM EDTA). Intact chloroplasts were collected by centrifugation at 700 × g for 30 s at 4°C and washed once with 10 ml of MCB2. The pellet (~300-μl packed volumes) was resuspended in 1.2 ml of cold extraction buffer (30 mM HEPES-KOH, pH 7.7; 10 mM magnesium acetate; 2 M KCl; 2 mM dithiothreitol) containing 0.2% Triton X-100. After 30 min incubation on ice, the lysate was centrifuged at 15 000 × g for 10 min. The supernatant (~1 ml) was dialyzed for 5 h against 100 ml of dialysis buffer (30 mM HEPES-KOH, pH 7.7; 3 mM magnesium acetate; 45 mM potassium acetate; 30 mM ammonium acetate; 10% glycerol). All steps were carried out at 0 to 2°C. The resulting green extract (10 to 20 μg of protein/μl) could be stored at −80°C at least for 2 months.

In vitro RNA editing reactions. 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, 1 mM ATP, 2 mM dithiothreitol, 1% Polyethylene Glycol 6000, 5% glycerol, 60 U of RNase inhibitor (TaKaRa), 1× proteinase inhibitor mixture (Complete; Boehringer Mannheim), 1 fmol of [³²P]mRNA substrate, and chloroplast extract (250 μg of protein). After incubation at 28°C for 2 h, the substrate mRNA was extracted once with phenol-chloroform and precipitated in 2 M ammonium acetate and 80% ethanol. The RNA was dissolved in 8 μl of water and digested into 5' mononucleotides with 1 μg of nuclease P1 (Wako) in the presence of 50 mM ammonium acetate (pH 4.8) at 37°C for 3 h. Mononucleotides were separated on cellulose thin-layer chroma-

tography (TLC) plates (20 by 20 cm; Funakoshi) by using isopropanol-HCl-water (25:24:1). The separated ³²P-labeled mononucleotides were visualized and quantified by using a Bioimaging Analyzer BAS2000 (Fuji Photo Film Co.).

Competition and UV cross-linking assays. Competitor RNAs were identical to the upstream and downstream regions of the mRNA substrates. A control RNA of 149 nt was synthesized (by using a T3 Megascript RNA synthesis kit) from the PCR product that was amplified from KS with T3 and T7 primers. One femtomole of [³²P]mRNA substrate and an indicated amount of competitor RNA were mixed and then incubated with chloroplast extract as described above. Samples for UV cross-linking were prepared by incubating 10 fmol mRNA (labeled with ³²P at the center of the *cis*-element) under in vitro editing conditions (25 μl, 28°C for 1 h). Reaction mixtures (in open 1.5-ml tubes on ice) were irradiated with UV light (254 nm, 1.8 J/cm²) by using a Funacrosslinker (Funakoshi Co.). This step was followed by digestion of the RNA with RNase A at 37°C for 1 h. Protein samples were dissolved in an equivalent volume of 2× loading buffer, followed by separation by 12.5% PAGE containing 0.1% sodium dodecyl sulfate. The separated proteins were visualized as described above.

Determination of editing sites and preparation of extracts from pea chloroplasts. Pea plants (*Pisum sativum* var. Alaska) were grown for 2 weeks under the same conditions as were described for the tobacco plants. The DNA and RNA were prepared as previously described (51). cDNA synthesis, PCR analysis, and direct DNA sequencing were carried out as previously described (29). Pea chloroplasts were prepared from young leaves from the upper part of the plants. The extraction method was identical to that described for tobacco. Reaction conditions were also identical, except that a fourfold amount of RNase inhibitor was added (240 U). The green extract (10 to 20 μg of protein/μl) could be stored at −80°C for at least 2 months.

RESULTS

Improvement of in vitro RNA editing extracts. Previously, we developed an in vitro RNA editing system from tobacco chloroplasts with *psbL* mRNA as a model substrate (31). This system yielded relatively high RNA editing activity for *psbL* mRNAs but not for *ndhB* (site IV) mRNAs. To improve the versatility of this system and to broaden its uses with a wide variety of chloroplast mRNAs, the procedure was modified in order to prepare more active extracts. It was found that extracts that were prepared quickly yielded better results. Consequently, chloroplast debris was removed from chloroplast lysates by short centrifugation. The extract thus obtained was green, whereas the protein concentration was similar to that of the original extract. To increase the sensitivity of RNA competition experiments, the amounts of mRNA substrates were reduced. The improved chloroplast extracts and reaction conditions resulted in RNA editing reactions that continued linearly for up to 2 h, three times longer than the previous method.

In vitro RNA editing of *psbE* and *petB* mRNAs. In the present study, we chose the RNA editing sites of *psbE* and *petB* mRNAs from tobacco chloroplasts since these transcripts are relatively abundant (13, 51); hence, their editing activities were expected to be high. The *psbE* gene encodes cytochrome *b*₅₅₉ and is located in the first gene position of the *psbE* gene cluster in the tobacco chloroplast genome (44, 54). The *petB* gene that encodes cytochrome *b*₆ is located within the *psbB* operon and is split by a 759-bp intron (51). Both genes have single C-to-U editing sites as shown in Fig. 1A. The mRNA substrates specifically labeled at these editing sites were prepared as previously described (31). The 5' end of the downstream region (+1 to +11; +1 represents the C that is edited) in the *psbE* mRNA was labeled with ³²P and joined with the upstream region (−128 to −1, Fig. 1A). The resulting mRNA substrate was incubated with the improved chloroplast extract prepared as described above. After incubation at 28°C for 2 h, RNA was

FIG. 1. In vitro RNA editing of *psbE* and *petB* mRNAs. (A) Schematic representation of tobacco chloroplast *psbE* and *psbB* operons and of mRNA substrates. The *petB* gene contains a single intron (shaded area). The 72nd codon in the *psbE* mRNA and the 204th codon in the *petB* mRNA are altered by RNA editing. The mRNA substrates include a 5' extension of 20 nt and a 3' extension of 17 nt (rectangular boxes). Asterisks indicate ^{32}P -labeled C residues to be edited. (B) Detection of in vitro RNA edited products from *psbE* and *petB* mRNAs. A total of 1 fmol each of the RNA substrates was incubated with or without chloroplast extracts for 2 h. After reactions, RNA was digested with nuclease P1, and the resulting mononucleotides were separated by TLC. U, marker pU; +Ex and -Ex, with or without chloroplast extracts, respectively.

isolated, digested with nuclease P1, and separated by cellulose TLC. As can be seen in Fig. 1B, a prominent U spot was detected in the TLC pattern, showing that C-to-U conversion occurred efficiently in vitro. The *petB* mRNA substrate, which includes the 121-nt upstream and 11-nt downstream sequences, was examined as described above, and a significant U spot was also detected.

The above substrate mRNAs possess 5' and 3' extensions derived from vector sequences (see Fig. 1A). After the editing

FIG. 2. Competition analysis with upstream and downstream mRNA parts. (A) *psbE* mRNA; (B) *petB* mRNA. Schematic structures of mRNA substrates and corresponding competitor RNAs are shown above TLC patterns. Increasing amounts of upstream RNAs (pE5 and pB5), downstream RNAs (pE3 and pB3), and a control RNA (a 149-nt sequence derived from vector) were added to in vitro editing reactions with *psbE* mRNA or *petB* mRNA; 10 fmol (lanes 4, 7, and 10), 100 fmol (lanes 5, 8, and 11), and 1000 fmol (lanes 6, 9, and 12). Asterisks indicate ^{32}P -labeled C residues to be edited. U, marker pU; -Ex, without chloroplast extract; 0, without competitor.

reaction, cDNA amplified from the isolated mRNA substrate by reverse transcription-PCR with primers complementary to the extensions yielded a single band. Sequence analysis of the cloned cDNAs revealed that C-to-U conversion, if any, occurred exclusively at the authentic editing sites in nine cDNAs of the 19 *psbE* clones examined and in four cDNAs of the 17 *petB* clones. This confirmed that the improved in vitro system also supports accurate RNA editing of the *psbE* and *petB* mRNAs.

Requirement of upstream sequences for editing. In tobacco chloroplasts, it has been suggested that RNA editing of *psbL* and *rpoB* (site II) mRNAs required depletable site-specific *trans*-acting factors (14, 15, 40). Recent in vitro analysis has unambiguously revealed the existence of such factors interacting with the corresponding upstream *cis*-elements (31).

To examine whether this is also the case for *psbE* and *petB* mRNAs, competition analysis with RNA molecules corresponding to upstream and downstream regions of the editing sites was carried out. As shown in Fig. 2, editing of the *psbE*

FIG. 3. In vitro analysis with mutated competitor RNAs and mutated mRNA substrates. (A) Schematic representation of altered regions constructed by scanning mutagenesis. The top sequence represents wild-type mRNA 5' proximal regions, and mutated parts are shown below with mutant names (m1 to m8). (B) Competition analysis with mutated competitor RNAs (m1 to m8) and a control RNA (ctr, as in Fig. 2). A total of 1 fmol each of wild-type mRNA substrates was reacted with 1,000 fmol each of the mutated competitor RNAs (20-nt 5' extension + a 121/128-nt upstream region). (C) Editing activity of mutated mRNA substrates. A total of 1 fmol each of mutated mRNAs was reacted. U, marker pU; -Ex, without chloroplast extract; 0, without competitor.

mRNA was substantially inhibited by a 100-fold molar excess of the upstream RNA competitor pE5 (lane 5). The competitor sequence consists of a 20-nt vector-derived sequence and a 128-nt *psbE* RNA portion (identical to the upstream part of *psbE* mRNA substrates). The addition of a 1,000-fold molar excess of pE5 abolished editing completely (lane 6). However, the downstream competitor pE3, which consists of a 17-nt vector-derived sequence and an 11-nt *psbE* sequence (identical to the downstream part of *psbE* mRNA substrates), did not affect the editing activity, even though a 1,000-fold molar excess was added (lane 9). The pE5 competitor is severalfold longer than pE3; hence, the possibility that inhibition depends on the length of competitor RNA can be raised. A control RNA, representing a 149-nt vector-derived sequence, hardly affected editing, although nonspecific inhibition was sometimes observed by a 1,000-fold molar excess (lanes 10 to 12), ruling out the above possibility. We concluded, therefore, that the upstream region in the *psbE* mRNA carries the *cis*-acting element. Furthermore, it is proposed that a *trans*-acting factor interacts with the element. Similar experiments were also performed for the *petB* mRNA substrate. It was found that only the upstream competitor pB5 inhibited editing (Fig. 2B), indicating that the *cis*-acting element of *petB* mRNA also resides in the upstream region.

Determination of *cis*-acting elements for editing. *cis*-Acting sequences necessary for editing have been reported to be located between -16 and +5 in the *psbL* mRNA (15) and between -42 and -2 in the *ndhB* mRNA (sites IV and V) (4). Therefore, it is expected that upstream sequences up to 40 nt long include *cis*-acting elements in other editing sites. A series of mutated competitor RNAs were constructed by scanning mutagenesis in which each successive 5-nt block in the region from -40 to -1 was substituted with its complementary nucleotide sequence (Fig. 3A). These sequences were used for competition analysis to define *cis*-acting elements involved in *psbE* and *petB* mRNA editing. The rationale behind this method rests on the premise that editing should be observed even in the presence of excess amounts of competitors with mutated *cis*-elements since they would be unable to trap putative *trans*-acting factors. Editing reactions were carried out as described above with a 1,000-fold molar excess of each competitor. As shown in Fig. 3B, the editing of *psbE* mRNA was inhibited by the addition of competitors m1 to m5 as effectively as the wild-type RNA, but not by the addition of m6 and m7. This result indicates the existence of a *trans*-acting factor that binds to the 10-nt sequence from -15 to -6 of *psbE* mRNA. Interestingly, m8 repressed editing as efficiently as did the wild-type competitor. This indicates that the 5-nt sequence

FIG. 4. Cross-competition analysis between *psbE* and *petB* mRNAs. Increasing amounts of competitors pE5 and pB5 (see Fig. 2) were added to the editing reactions with *psbE* and *petB* mRNAs: 100 fmol (lanes 4 and 6) and 1,000 fmol (lanes 5 and 7). U, marker pU; -Ex, without chloroplast extract; 0, without competitor.

immediately upstream from the editing site is not needed for the binding of the *trans*-acting factor. In the case of *petB* mRNA, the addition of competitors m5 to m7 did not exhibit any inhibition, whereas m8 significantly repressed editing, indicating again that a *trans*-acting factor binds to the 15-nt sequence between -20 and -6.

To examine whether these sequences are sufficient for the editing reaction, we performed *in vitro* assays with a series of mRNA substrates whose upstream sequences (between -40 and -1) were mutated by the same manner. As shown in Fig. 3C, editing of the *psbE* mRNA substrates m1 to m5 occurred at a level comparable to that of wild-type mRNA, whereas no editing was observed with the m6 to m8 substrates. This result clearly shows that sequences located between -15 and -1 are required for the editing of *psbE* mRNA. Since the entire binding site of the *trans*-acting factor resides within the region from -15 to -6, the results of the present experiment indicated that the editing reaction requires an additional sequence located between the -5 and -1 positions. This is consistent with the observation that changing A at -1 to C abolished the editing of *psbL* mRNAs (15). A similar experiment was performed with *petB* mRNA where the m1 to m4 mRNA substrates were edited but not the m5 to m8 substrates (Fig. 3C). This finding suggests that the editing of *petB* mRNA also requires the *trans*-factor binding site and adjacent sequences located between positions -5 and -1.

Detection of site-specific protein factors. To examine whether distinct *trans*-factors interact with the *cis*-acting elements of *psbE* and *petB* mRNAs, cross-competition experiments were carried out with heterologous competitor RNAs. As shown in Fig. 4, the editing of *psbE* mRNA was inhibited by its own upstream competitor pE5 (as described before) but not by the *petB* upstream competitor pB5. Conversely, the editing of *petB* mRNA was only inhibited by pB5 and not by pE5. Taken together, these results indicate that each *cis*-acting element is bound by a distinct factor.

The factors described above are likely to be proteins, since the interaction of a protein (p25) has been demonstrated for the *cis*-element of *psbL* mRNA (31). In order to detect protein species binding to the defined *cis*-acting elements, UV cross-linking experiments were carried out with mRNA substrates which were specifically labeled at the center of the *cis*-ele-

FIG. 5. Detection of tobacco chloroplast proteins bound to the *cis*-acting elements of *psbE* and *petB* mRNAs by UV cross-linking. The *psbE* and *petB* mRNAs with 32 P labels at the center (asterisks) of the *cis*-acting elements (underlined) were used as substrates. A total of 10 fmol each of the mRNAs was incubated for 1 h at 28°C in the editing reaction mixture and then UV irradiated (254 nm). The mixture was treated with RNase A, followed by sodium dodecyl sulfate-12.5% PAGE. A total of 1,000 fmol each of the competitors was added. -Ex, without chloroplast extracts; 0, without competitor. Protein size markers are shown at the left (Rainbow; Amersham).

ments: C at -10 in *psbE* mRNA and U at -13 in *petB* mRNA (Fig. 5). Moreover, upstream competitors were added to differentiate between specific and nonspecific binding. Using *psbE* mRNA (lane 2), several protein bands of 56 kDa and of 28 to 33 kDa were detected. When an excess amount of pE5 competitor was added, the 56-kDa band completely disappeared, whereas the 28- to 33-kDa bands were still present (lane 3). This observation was specific for pE5, since pB5 did not change the band pattern (lane 4). These results indicate that the 56-kDa protein (p56) binds specifically to the *cis*-acting element of *psbE* mRNA. Thus, p56 is the site-specific protein factor necessary for the editing of *psbE* mRNA.

In the case of *petB* mRNA, a large amount of 28- to 33-kDa proteins, in addition to a sharp 70-kDa band, was detected (lane 6). Only the 70-kDa protein (p70) disappeared with pB5 (lane 7) but not with pE5 (lane 8). This result confirms the notion that p70 is the site-specific protein factor necessary for the editing of *petB* mRNA. Compared to the cross-linking pattern observed with *psbE* mRNA (lanes 2 to 4), additional proteins and a high level of 28- to 33-kDa proteins were found to be nonspecifically cross-linked with *petB* mRNA substrates (lanes 6 to 8). This is probably because the efficiency of cross-linking is dependent on the nature of the RNA sequences used.

Editing of *psbE* and *petB* mRNAs in pea chloroplasts. A total of 20 to 30 editing sites have thus far been reported in chlo-

FIG. 6. In vitro RNA editing of *psbE* and *petB* pre-mRNAs with the pea chloroplast extract. (A) Comparison of sequences surrounding the editing sites in tobacco and pea *psbE* and *petB* pre-mRNAs (un-edited). (B) In vitro edited products from *psbE* and *petB* mRNAs with the pea chloroplast extract. The reaction conditions were as in Fig. 1B. U, marker pU; +Ex and -Ex, with or without chloroplast extract, respectively.

roplast transcripts from angiosperms, with a portion of these editing sites being conserved between species (16, 30, 52). We chose to study the pea plant, a dicotyledonous plant that diverged from the tobacco lineage ~100 million years ago (57), to examine whether the conservation of editing sites between species correlated with that of the site-specific *trans*-acting factors. In pea chloroplasts, the DNA sequence corresponding to the editing site of tobacco *psbE* mRNA (Ccu [Pro]) is Tct (Ser) (56). Therefore, no editing is required at this site (see Fig. 6A). On the other hand, the cCa (Pro) codon at the editing site of tobacco *petB* mRNA is conserved in the pea genome (34). The Leu codon (cUa) at this position is conserved among many species. Maize, rice, and tobacco restore the Leu from Pro codons by RNA editing (16, 21). Furthermore, the conversion of Leu at this position to Pro was found to abolish cytochrome *b₆* activity in *Chlamydomonas* spp. (60). Therefore, the Leu codon is also likely to be restored from the Pro codon by RNA editing in pea chloroplasts. Direct sequencing of DNA and RNA isolated from pea seedlings showed that the cCa sequence present in the chloroplast DNA was converted to cTa in the cDNA. This confirmed that RNA editing of *petB* mRNA occurs also in pea plants (data not shown).

An in vitro RNA editing system from pea chloroplasts. To compare *cis*-elements and *trans*-factors between tobacco and pea plants, an in vitro RNA editing system was developed from pea chloroplasts. Intact chloroplasts were prepared from 2-week-old seedlings, and the extraction followed the method used for tobacco chloroplasts. Tobacco mRNA substrates were used since the coding regions of pea and tobacco chloroplast genes are highly conserved (*psbE*, ~95% identity; *petB*, ~90%

FIG. 7. Competition analysis of *petB* mRNA editing with the pea extract. (A) Competition analysis with 1,000 fmol each of upstream (pB5), downstream (pB3) and control competitor RNAs (see Fig. 2). (B) Competition analysis with mutated competitor RNAs (pB5 m1 to m8, see Fig. 3). U, marker pU; -Ex, without chloroplast extract; 0, without competitor; ctr, control RNA.

identity). As shown in Fig. 6A, the *cis*-sequence of tobacco *psbE* mRNA is completely conserved in the pea, and that of tobacco *petB* mRNA shows complete identity with the pea except for 1 nt (the -17 position is a U in tobacco plants but a C in pea plants).

Reaction conditions were the same as those for tobacco, except a fourfold amount of RNase inhibitor was added since RNase activity is relatively high in pea chloroplast extracts. Incubation of the *psbE* mRNA with the pea chloroplast extract did not induce any change (Fig. 6B, lane 3). This indicated that the editing activity for *psbE* mRNAs was lost in pea chloroplasts probably because the gene already possesses a T residue at this position. On the other hand, the tobacco *petB* mRNA substrate yielded a U spot resulting from the editing reaction with the pea extract. This confirms that pea chloroplasts possess the editing machinery for *petB* mRNAs but not for *psbE* mRNAs. Additionally, this result demonstrated that the improved procedure for preparing tobacco chloroplast extracts could be directly applied to another plant species.

***cis*-Acting element of *petB* mRNA in pea chloroplasts.** To examine the presence of factors necessary for *petB* mRNA editing in the pea extract, competition experiments were performed with a 1,000-fold excess of upstream, downstream, and control RNA competitors (pB5, pB3, and control RNA). As shown in Fig. 7A, pB5 inhibited editing effectively (lane 4), whereas pB3 and the control RNA showed no inhibition (lanes 5 and 6). This indicates that a *trans*-acting factor interacts with the upstream sequence. To define the *cis*-acting element, a similar analysis was carried out with mutated pB5 competitors prepared for tobacco extracts (see Fig. 3). The observed results were the same as those found with the tobacco extracts (see Fig. 3B). Competitors m5 to m7 did not reduce editing activities as well as did the control RNA, whereas competitors m1 to m4 and m8 inhibited editing to a degree similar to that of

FIG. 8. Detection of pea chloroplast proteins bound to the *cis*-acting elements of tobacco *psbE* and *petB* mRNAs by UV cross-linking. Assays were as in Fig. 5.

the wild-type RNA (Fig. 7B). Thus, sequences essential for editing in the pea plant reside within the region from positions -20 to -6 . This is identical to situation that prevails with the tobacco editing machinery. It should be noted that although the position of the *cis*-acting element is identical between the tobacco and pea plants, 1 nt (at position -17) is different (U in tobacco, C in pea). This suggests that although the location of *cis*-elements appears to be quite rigid, minor changes in sequence can be tolerated by the cognate *trans*-factors.

Site-specific protein factors in pea chloroplasts. In an effort to detect specific proteins in the pea extract, UV cross-linking experiments were performed with tobacco mRNA substrates (labeled at -10 in *psbE* mRNA and at -13 in *petB* mRNA) and corresponding competitor RNAs (pE5 and pB5). Using *psbE* mRNA as substrate, a broad band of ca. 32 kDa was visible in the absence of competitors, whereas homologous (pE5) and heterologous (pB5) competitors did not essentially affect the band pattern (lanes 2 to 4). This indicates that no proteins specifically bind to the *cis*-acting element of tobacco *psbE* mRNA (Fig. 8). Based on this result, together with the absence of editing activity observed with the *psbE* mRNA (mentioned above), we concluded that the *trans*-acting protein required for the editing of *psbE* mRNAs is absent in pea chloroplasts. On the other hand, several additional proteins were detected with the *petB* mRNA substrate besides the fast-migrating proteins observed in the absence of competitors (lane 6). The addition of the pB5 competitor, but not pE5, resulted in the specific disappearance of the 70-kDa protein (p70, lanes 7 and 8). This indicates p70 is the site-specific protein for the editing of *petB* mRNA in pea chloroplasts. The molecular mass (p70) of the tobacco and pea *petB* mRNA-specific proteins is the same, suggesting that the *trans*-acting factors are conserved along with their requisite *cis*-acting elements.

DISCUSSION

A total of 20 to 30 RNA editing sites have been reported in transcripts from higher-plant chloroplasts. The recognition of

these sites (ca. 1 C residue out of every 1,000 C residues in whole transcripts) should be extremely accurate and efficient. During the past several years, tobacco plants containing transgenic chloroplasts have facilitated the *in vivo* analysis of elements involved in RNA editing. However, the *in vivo* technique is time-consuming and possesses limitations in terms of the biochemical analysis of editing processes. An *in vitro* RNA editing system was recently developed from tobacco chloroplasts (31), in which the site-specific labeling of C residues to be edited enabled us to detect accurate editing events even at a low level. Here we simplified the original procedure for preparing chloroplast extracts, and the improved *in vitro* system is more active than the original one so that a wide range of chloroplast mRNAs can now be analyzed. Moreover, this improved method is likely to be a versatile procedure in the preparation of active extracts from a variety of higher plants. This methodology has been successfully applied to pea (described below). We also optimized reaction conditions by which highly sensitive assays can be made. The improved *in vitro* system thus represents a powerful tool for exploring functional elements and factors involved in site recognition and base conversion reactions.

Using the above system, we have defined the *cis*-acting elements, which are sequences necessary for the binding of *trans*-acting factors, for the editing of tobacco *psbE* and *petB* mRNAs. These elements reside in similar upstream regions from the editing sites, i.e., between positions -15 and -6 in *psbE* mRNAs and between positions -20 and -6 in *petB* mRNAs. The *cis*-acting elements of several other tobacco chloroplast mRNAs were characterized by using *in vivo* transplastomic techniques. A 22-nt sequence located between -16 and $+5$ is sufficient to direct efficient *psbL* mRNA editing (15). The sequence located between positions -12 and -2 is necessary for the editing of *ndhB* mRNA (site IV) and between positions -21 and -11 for the editing of site V (4). In the case of the *rpoB* mRNA (site II), a 27-nt sequence located between positions -20 and $+6$ is sufficient to allow editing (41). Based on the available information, most *cis*-acting elements for RNA editing in chloroplasts are located upstream of the editing sites and are ~ 10 nt long. This finding is consistent with the observation that the editing site of *ndhB* mRNA (site V) is defined by the distance from its upstream *cis*-element (26).

It is unlikely that regions further upstream from position -40 contain additional *cis*-acting elements, at least those involved in site-recognition for the editing of *psbE* and *petB* mRNAs. Excess amounts of competitor mRNAs with mutated *cis*-elements did not inhibit editing, in spite of them having upstream regions identical in size and sequence to wild-type mRNA substrates (see Fig. 3B). However, it has been reported that the region between positions -42 and $+42$ is not sufficient for editing of *ndhB* mRNAs (sites II and III) (4), suggesting that a different type of *cis*-element, one involved in editing efficiency, might exist far from the editing sites.

In vitro editing assays with mutated mRNA substrates clearly indicated that the *cis*-elements (*trans*-factor binding sites) described above are not sufficient and that the 5-nt region immediately upstream from the editing sites is essential for editing (see Fig. 3C). It is unclear at present why sequences located between positions -5 and -1 are necessary for the editing reactions. A putative cytidine deaminase may require

these sequences, in which at least a pyrimidine at position -1 is likely to be critical for the editing of most mRNAs, since 29 of 31 tobacco editing sites include pyrimidines at this position (30). Moreover, editing of *ndhB* mRNAs (site V) was impaired if the U at position -1 was converted to a G (4). Thus, our observation that mutated mRNAs, *psbE* m8 and *petB* m8, could not be edited efficiently can be explained by the replacement of pyrimidines by purines at position -1 . However, only 2 (*psbL* and *ndhD* mRNAs) of 31 tobacco editing sites are the exception, with an A residues at position -1 , thus generating AUG initiation codons from ACG as a result of editing. Furthermore, tobacco *psbL* mRNA, whose position at -1 is A, was not edited when the A residue was substituted by a C residue (15). Thus, it is possible that there are at least two distinct deaminases: one preferring pyrimidines at -1 , while the other displays a preference for A residues. Alternatively, site-specific *trans*-acting factors may possess deamination activity.

Using a pair of sample mRNAs (*psbE* and *petB*), we unambiguously showed that a *cis*-acting element is recognized by a unique protein: p56 for *psbE* mRNA and p70 for *petB* mRNA. It is suggested that a unique RNA-binding protein recognizes each editing site in chloroplasts. The tobacco chloroplast genome does not have a sufficient number of unknown ORFs correspond to the 25 to 30 proteins (54); hence, the *trans*-acting factors are most likely to be imported from the cytoplasm. This is the first report for the biochemical existence of multiple site-specific RNA-binding proteins involved in mRNA editing. Two systems have been extensively studied for the editing of multiple sites. One is directed by the secondary structure of RNAs for A-to-I conversion in mammalian cells (20), and another is directed by guide RNAs involved in the insertion and/or deletion of U's in *Trypanosoma* kinetoplastids (47). The system for site recognition of chloroplast RNA editing may be similar to that of *apoB* mRNAs, which is known to be directed by a sequence-specific RNA-binding protein (19). Although the nature of these proteins is unknown, they should have at least two domains in common: one for the recognition of a ~ 10 -nt *cis*-element and the other for the contact site with a catalytic factor or the catalytic domain itself to facilitate the deamination of C residues to be edited.

In angiosperm chloroplasts, the number of editing sites reported so far number ~ 30 . These editing sites are not well conserved (see, for example, reference 52). For example, only 12 sites are conserved between *N. tabacum* (a total of 31 sites) and *Zea mays* (a total of 27 sites). This indicates that a substantial number of editing sites are species specific. Therefore, it is interesting to consider whether the homologous editing factor can operate if an editing site is conserved between different plant species. Since the chloroplast genome sequences from land plants are highly conserved (see, for example, reference 55), the presence or absence of *cis*-acting elements cannot be inferred by simple comparison of sequences surrounding editing sites between edited and unedited species. Chloroplast transformation techniques have been used to dissect *cis*-elements but available practically only for tobacco (8, 50). To compare biochemical mechanisms of RNA editing between species, we prepared an additional in vitro editing system from pea chloroplasts (see above). In pea extracts, the editing of *petB* mRNAs was similar to that of tobacco extracts (see Fig. 6). Additionally, the *cis*-acting element of *petB*

mRNA was found to be identical to tobacco. Surprisingly, UV cross-linking experiments indicated that the molecular mass of the pea site-specific RNA-binding protein is also the same as for tobacco (70 kDa). These results suggest that editing factors involved in site recognition (*cis* and *trans*) are conserved between species.

It was reported that the editing sites of spinach *psbF* mRNA and maize *rpoB* mRNA (site IV) were not edited in transgenic tobacco plants in which no corresponding site is present (3, 40). However, tobacco was found to retain the editing activity of *ndhA* mRNA (site I) despite the absence of the target site (43). The pea codon corresponding to the tobacco *psbE* editing site (Pro [Ccu], which is restored to Ser [Ucu] upon RNA editing) is already Ser (Tct) at the DNA level. This requires no RNA editing to produce the conserved protein. In fact, no editing activity was detected for the *psbE* mRNA with Ccu in the pea chloroplast extract. Moreover, we found no protein which specifically bound to the *cis*-acting element of *psbE* mRNA. Our in vitro analysis clearly revealed that the loss of editing activity is due to the absence of the corresponding *trans*-acting factor (in this case p56). Hence, coevolution of an editing site and its cognate *trans*-acting factor was demonstrated biochemically at least in the case of *psbE* mRNA editing among tobacco and pea. Our results support the idea that higher plants possess a set of sequence-specific RNA-binding proteins for the recognition of each editing site in a species-specific manner.

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