The EMBO Journal vol.15 no.21 pp.5958–5964, 1996

Sequences directing C to U editing of the plastid *psbL* mRNA are located within a 22 nucleotide segment spanning the editing site

Sumita Chaudhuri and Pal Maliga[1]

Waksman Institute, Rutgers, The State University of New Jersey, PO Box 759, Piscataway, NJ 08855-0759, USA

[1]Corresponding author

This paper is dedicated to Hans Kössel (1934–1995), a colleague and a friend.

In plastids, editing of an ACG codon to an AUG codon creates the translation initiation codon for the *psbL* and *ndhD* transcripts in tobacco. To identify the RNA segment required for *psbL* editing, chimeric kanamycin resistance genes were constructed containing *psbL* deletion derivatives, and tested *in vivo* for editing in transgenic plants. We report here that a 22 nucleotide segment is sufficient to direct efficient *psbL* editing, including 16 nucleotides upstream and five nucleotides downstream of the editing site. Mutation of the A nucleotide to a C upstream of the editing site completely abolished editing, while mutation of the downstream G to a C only reduced the editing efficiency. Out of the 22 nucleotide editing target sequence, the 16 upstream nucleotides were found to compete with the endogenous *psbL* transcript for a depletable *trans*-factor. To test whether editing of initiation codons involves a common *trans*-factor, a chimeric gene containing the *ndhD* editing site was expressed in tobacco plastids. As for *psbL*, editing of the *ndhD* site requires a depletable *trans*-factor. However, the *ndhD trans*-factor is distinct from that required for *psbL* editing. Distinct *cis*-sequences and *trans*-factor requirements for the *psbL* and *ndhD* editing sites indicate an individual recognition mechanism for the editing of plastid initiation codons.

Keywords: mRNA editing/plastid *ndhD* gene/plastid *psbL* gene/tobacco plastid transformation

Introduction

RNA editing is a process that post-transcriptionally alters RNA sequences. It has been detected in divergent organisms including trypanosomes, *Physarum polycephalum*, mammals, viruses and higher plants involving widely different molecular mechanisms (reviewed in Chan, 1993; Gray and Covello, 1993; Benne, 1994; Simpson and Thiemann, 1995; Innerarity *et al.*, 1996). In higher plants, editing of plastid and mitochondrial RNAs involves C to U conversions and rare cases of U to C changes in mitochondria. For reviews on RNA editing in plastids and plant mitochondria see Bonnard *et al.* (1992), Gray and Covello (1993), Kössel *et al.* (1993), Schuster and Brennicke (1994), Maier *et al.* (1995) and Hanson *et al.* (1996). The number of editing sites in plastids is estimated to be ~25 (Maier *et al.*, 1995) while in plant mitochondria it is 1000 or more (Schuster and Brennicke, 1994). In spite of RNA editing being such a widespread phenomenon in plants, very little is known about the molecular details of the plant organellar editing process. Comparison of sequences surrounding editing sites has failed to identify any conserved primary sequence and/or structural motifs that could direct the site-selection process. The recent development of an *in vitro* editing system should lead to accelerated progress in the analysis of RNA editing in plant mitochondria (Araya *et al.*, 1992; Yu and Schuster, 1995). Although an *in vitro* system for editing in plastids is still lacking, the availability of plastid transformation allows an *in vivo* approach to study plastid editing (Bock *et al.*, 1994; Bock and Maliga, 1995; Sutton *et al.*, 1995).

Previously, we reported that in tobacco, a 98 nucleotide fragment spanning the *psbL* editing site contains all *cis*-sequences required for *psbL* editing (Chaudhuri *et al.*, 1995). Furthermore, we had reported that expression of the chimeric transcripts containing the 98 nucleotide *psbL* fragment led to a significant decrease in the editing efficiency of the endogenous *psbL* mRNA, but not of editing sites in other mRNAs. Reduced *psbL* editing in the transgenic plants was explained by competition of the *psbL* targeting sequence for a site-specific editing factor that is present in limiting amounts (Chaudhuri *et al.*, 1995). In this study, deletion derivatives of the 98 nucleotide fragment were expressed as parts of chimeric transcripts to define the *cis* sequences required for *psbL* editing. We report here that a 22 nucleotide fragment is sufficient to direct *psbL* editing. However, only 16 nucleotides competed for the *psbL*-specific editing factor.

In addition to *psbL*, editing was shown to create the AUG translation initiation codon for *ndhD* in tobacco (Neckermann *et al.*, 1994). To test whether editing of initiation codons involves a common depletable *trans*-factor, a chimeric gene containing the *ndhD* editing site was expressed in tobacco plastids. We have found that, as for *psbL*, editing of the *ndhD* site requires a depletable *trans*-factor. However, this *trans*-factor is distinct from that required for *psbL* editing.

Results

Defining the cis-sequences directing psbL editing

The *psbL* gene is part of the *psbE* operon which contains the *psbE*, *psbF*, *psbL* and *psbJ* reading frames (Carillo *et al.*, 1986). In a previous report (Chaudhuri *et al.*, 1995) we described editing of the *psbL* translation initiation site in a chimeric mRNA containing a 98 nucleotide Δ*psbF*/Δ*psbL* fragment (–63/+34 in plasmid pSC2, Figure 1A). In this chimeric construct the first open reading frame is a truncated *psbF* (Δ*psbF*) gene containing 40 nucleotides of the C-terminus. The second open reading frame con-

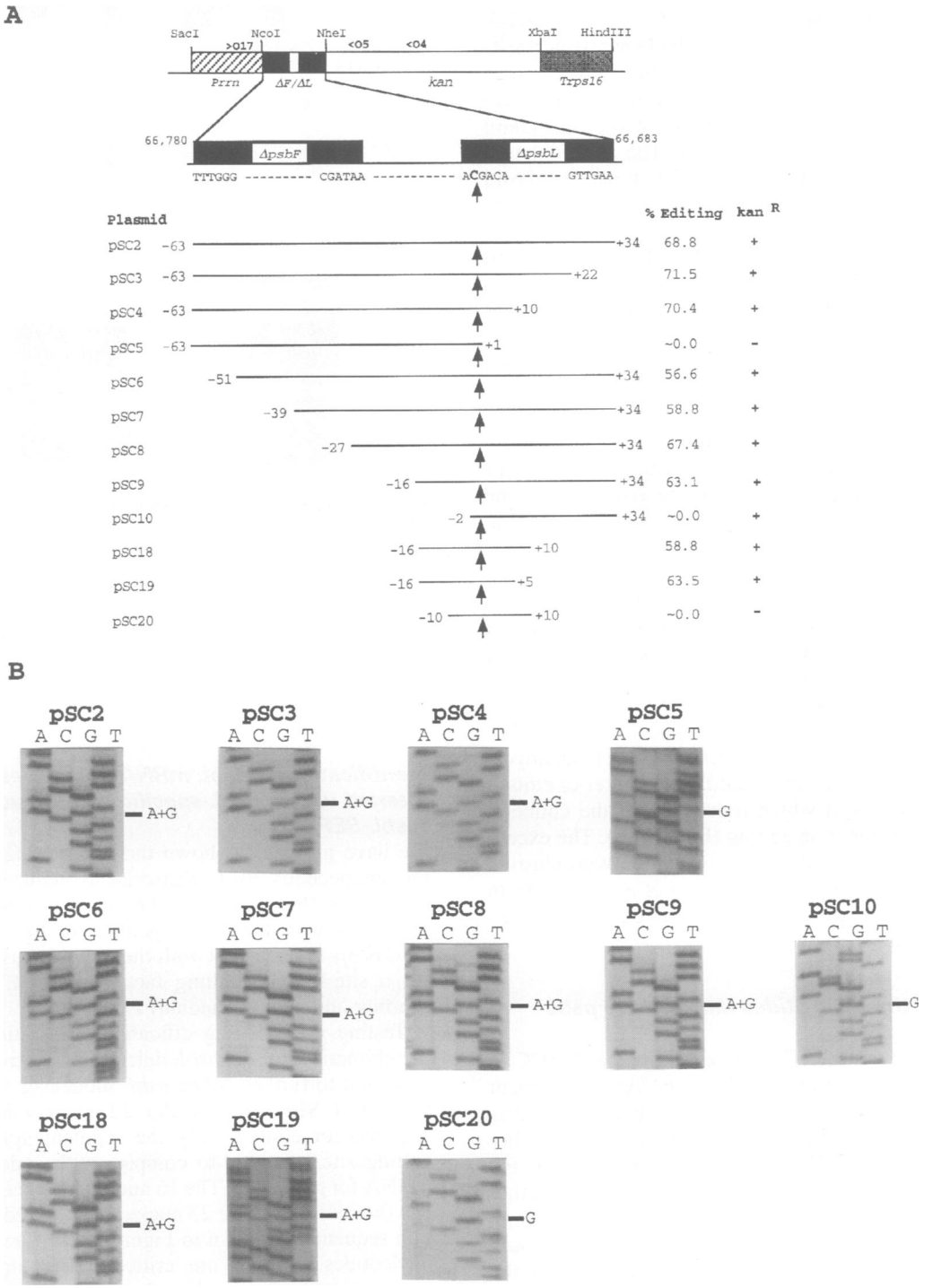

Fig. 1. Defining the region required for *psbL* editing. (**A**) The map of the chimeric Δ*psbL/kan* gene, with the 98 nucleotide Δ*psbF*/Δ*psbL* fragment enlarged, is shown at the top. The positions of primers O4, O5 and O17 are indicated. 66,780 and 66,683 are the nucleotides at the ends of the 98 nucleotide Δ*psbF*/Δ*psbL* fragment in the tobacco plastid genome (Shinozaki *et al.*, 1986). Below is the listing of pPRV111A plasmid derivatives which carry chimeric Δ*psbL/kan* genes. The nucleotide position at the end of the Δ*psbF*/Δ*psbL* deletion derivatives is given relative to the edited C (position 0; arrow). The efficiency of editing of the chimeric Δ*psbL/kan* mRNA (%) and the kanamycin resistance phenotype of the transgenic plants are listed. (**B**) Editing of the *psbL* site in the chimeric mRNAs. The cDNAs were PCR amplified with primers O17 and O4 and directly sequenced with primer O5. Due to the polarity of O5, the sequence shown is complementary to the mRNA. Accordingly, A at the edited position indicates a C to U conversion event and a G an unedited C nucleotide.

tained 36 nucleotides of the N-terminus of *psbL* (Δ*psbL*) translationally fused with the bacterial kanamycin resistance (*kan*) gene to yield the Δ*psbL/kan* fusion protein. The two open reading frames are separated by 22 nucleotides of intergenic region (Figure 1A).

To identify the sequences required for *psbL* editing, deletion derivatives of the 98 nucleotide Δ*psbF*/Δ*psbL* fragment were tested for editing *in vivo*. As before, the *psbL* deletion derivatives were fused N-terminally to a *kan*, and cloned in the plastid P*rrn*/T*rps16* expression cassette to create chimeric genes (Figure 1A). Thus, for all the constructs, translation of Δ*psbL/kan* was made

dependent on editing of the *psbL* ACG codon to AUG. Editing therefore could be tested by the kanamycin resistance phenotype. The only exception is the chimeric gene containing a –2/+34 fragment (in plasmid pSC10) where the initiation codon for the translation of the Δ*psbL/kan* reading frame was provided by P*rrn*. The *psbL* deletion derivatives were introduced into the tobacco plastid genome in plastid vector pPRV111A (Zoubenko *et al.*, 1994) by linkage to a selectable spectinomcyin resistance gene (Chaudhuri *et al.*, 1995). The pPRV111A vector targets insertions into the repeated region of the plastid genome, therefore each transgene is present in two copies per genome.

The upstream deletion series included constructs with 5′-ends at positions –63, –51, –39, –27, –16, –10 and –2 nucleotides relative to the editing site (position 0). The downstream deletion series included constructs with 3′-ends at positions +34, +22, +10, +5 and +1 nucleotides relative to the editing site. The editing efficiency of the chimeric mRNAs was determined by direct sequencing and PhosphorImager analysis of PCR-amplified cDNAs. Editing in the deletion derivatives was maintained as long as the constructs contained 16 nucleotides of upstream and five nucleotides of the downstream sequence relative to the editing site (Figure 1A and B). Interestingly, in the deletion series, the percentage of the chimeric mRNA that is edited (editing efficiency) was either similar to that of the full size 98 nucleotide Δ*psbF*/Δ*psbL* fragment (~50–70%) or barely detectable (~0%). Expression of kanamycin resistance was also a reliable qualitative marker of editing in all transformants in which translation of the chimeric mRNA was dependent on editing (Figure 1A). The exception were plants obtained by transformation with plasmid pSC10 in which kanamycin resistance is expressed from the translation initiation codon contained in the P*rrn* promoter fragment.

Mutation of the nucleotides flanking the psbL editing site

We changed the edited ACG codon to CCG and ACC in the efficiently edited 98 nucleotide Δ*psbF*/Δ*psbL* fragment to address the following issues. (i) Whether the flanking nucleotides are critical for editing. (ii) Whether the fidelity of editing the correct C is maintained when one of the flanking nucleotides is changed to a C. (iii) Whether translation initiation at this site is required for editing, since changing the ACG codon to CCG and ACC would eliminate the possibility of translation initiation at the edited codon.

Mutation of the upstream nucleotide (ACG to CCG; Nt-pSC14 line) resulted in the loss of editing (~0%; Figure 2). Mutation of the downstream nucleotide (ACG to ACC; Nt-pSC15 line) allowed editing at the correct C, but at a significantly reduced efficiency (~20%; Figure 2). The mutational analysis therefore indicated that the alteration of A to C directly upstream of the edited C is inhibitory to editing, while mutation of the downstream G residue to C is compatible with editing but is required for optimal efficiency. In addition, editing of the correct C in the mutated codon ACC points to a high fidelity mechanism of the editing apparatus in the choice of the editing site. Furthermore, editing of the ACC codon suggests that translation initiation at this codon is not required for editing.

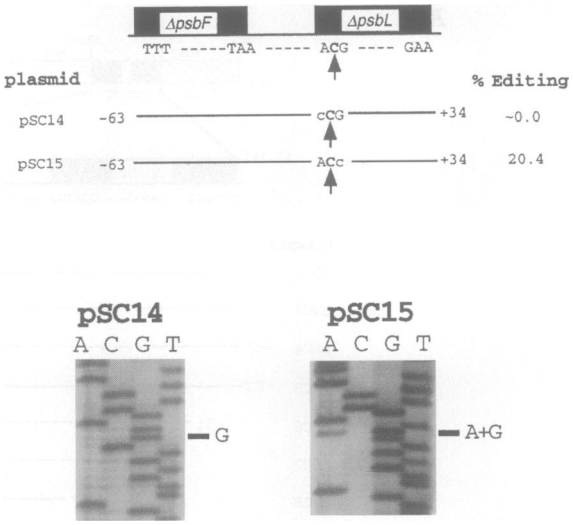

Fig. 2. Test of *psbL* editing in transgenic plants with mutations adjacent to the editing site. The position of the edited C (arrow) and the flanking nucleotides within the 98 nucleotide Δ*psbF*/Δ*psbL* fragment are shown at the top. Mutations in plasmids pSC14 and pSC15 are in lower case letters. Editing was tested by sequencing the chimeric cDNAs (bottom). Calculated editing efficiencies of the chimeric mRNA (%) are listed. For experimental details see legend to Figure 1.

Identification of psbL mRNA sequences which interact with a psbL-specific editing factor (psbL-SEF)

We have previously shown that the editing efficiency of the endogenous *psbL* transcript is reduced in plastids expressing the chimeric *psbL* mRNA. Reduced editing efficiency was due to competition of the 98 nucleotide Δ*psbF*/Δ*psbL* fragment with the endogenous *psbL* mRNA for a site-specific editing factor (*psbL*-SEF) present in limiting amounts (Chaudhuri *et al.*, 1995).

Testing *psbL* editing efficiency in plastids expressing the chimeric Δ*psbF*/Δ*psbL* deletion derivatives (Figure 1) was used to further define *psbL* sequences which interact with *psbL*-SEF. Out of the 22 nucleotides minimally required for editing, only the segment upstream of the editing site was able to compete with endogenous *psbL* mRNA for *psbL*-SEF. The 16 nucleotide *psbL*-SEF binding site (boxed) within the 22 nucleotide *psbL* editing recognition sequence is shown in Figure 3A. Sequences between nucleotides –16/–10 are critical for competition since competition is abolished in plastids containing the pSC20 construct which lacks this sequence (Figure 3B). Interestingly, the plants expressing the pSC14 construct with the A to C mutation at position –1 also maintained competition, although this mutation completely abolished editing. The *psbL* editing efficiency data for the critical constructs are shown in Figure 3A and B. While in the wild-type plants *psbL* mRNA is >99% edited, competition in the transgenic lines lead to accumulation of a significant amount of unedited *psbL* transcript.

Editing of the ndhD initiation codon in chimeric mRNA

Sequence analysis of *ndhD* and the corresponding cDNA by Neckermann *et al.* (1994) has established that the *ndhD*

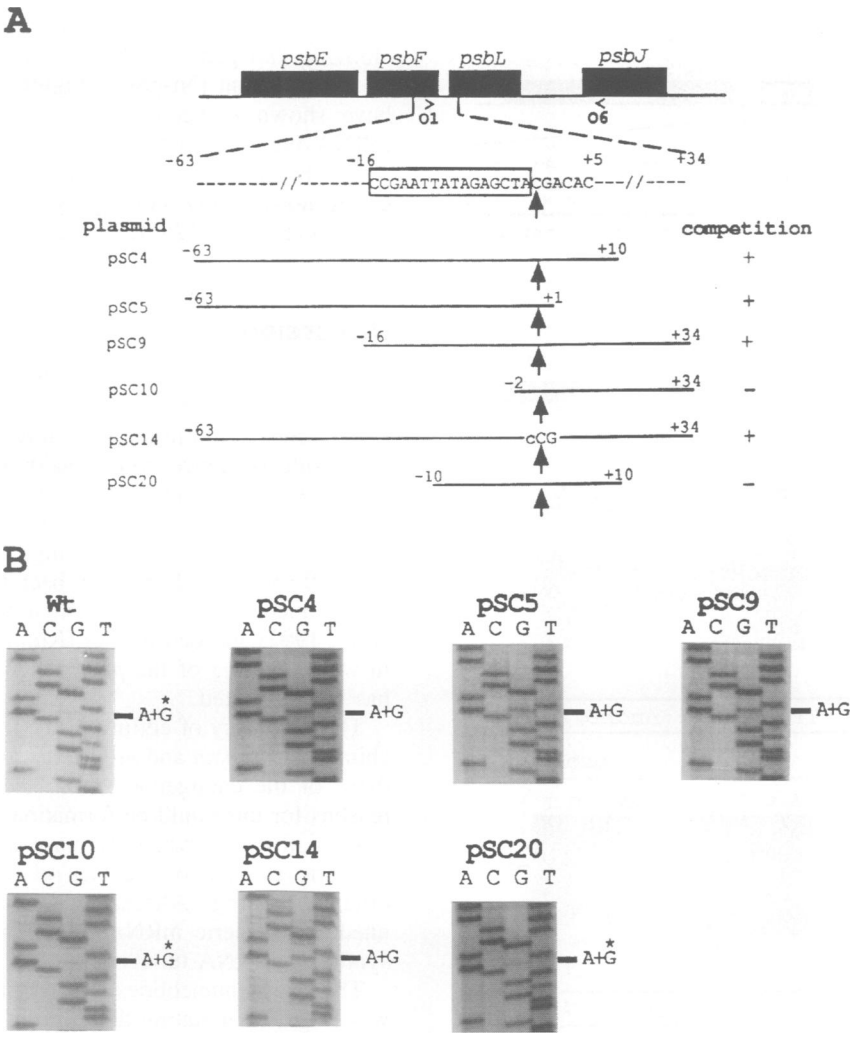

Fig. 3. Testing of competition for the *psbL*-specific *trans*-factor (*psbL*-SEF) in the transgenic plants. (**A**) The map of the *psbE* operon containing the *psbL* gene, with the position of oligonucleotides O1, O6 and O7 used for PCR amplification and sequencing. The 22 nucleotide (–16/+5) sequence required for editing is shown. The edited C is marked by an arrow. The 16 nucleotide segment competing for *psbL*-SEF is boxed. The plasmids used to obtain the transgenic plants are listed, as defined in Figures 1 and 2. Competition (+) was indicated by reduced editing efficiency of the *psbL* mRNA, as compared to non-transformed, wild-type plants. (**B**) Editing of *psbL* mRNAs. The cDNAs were PCR amplified with primers O1 and O6 and directly sequenced with primer O7. Due to the polarity of O7, the sequence shown is complementary to the mRNA. Accordingly, A at the edited position indicates a C to U conversion event, G an unedited C nucleotide. A+G* denotes nearly complete editing (>99%) as in the wild-type plants. A+G denotes partial editing of the *psbL* transcripts.

translation initiation codon is created by editing of an ACG codon to an AUG codon in tobacco, spinach and snapdragon. We set out to test whether *ndhD* editing requires a depletable *trans*-factor as found for *psbL*, and whether this *trans*-factor is utilized for the editing of both initiation codon sites. For this, an 89 nucleotide fragment (–48/+40) spanning the *ndhD* editing site was translationally fused with the *kan* coding region and cloned in a P*rrn*/T*rps16* expression cassette (Figure 4A and B). The chimeric gene was introduced into the tobacco plastid genome by linkage to a spectinomycin resistance gene in plastid vector pPRV111A. Transformation with this vector introduces two copies of the transgene per plastid genome (Zoubenko *et al.*, 1994). In the chimeric gene, expression of the Δ*ndhD*/*kan* fusion protein was dependent on the editing of the *ndhD* site. To prevent translation from an upstream AUG, a point mutation was introduced 26 nucleotides upstream of the editing site, changing an A to a C (underlined in Figure 4A).

Nt-pSC23 plants expressing the Δ*ndhD*/*kan* protein were resistant to kanamycin, indicating editing of the *ndhD* site. Direct sequencing of PCR amplified Δ*ndhD*/*kan* revealed a very low efficiency (~7%) of editing (Figure 4C). The *ndhD* transcript in the wild-type plants is edited at significantly higher efficiency (~45%), which is reduced in the Nt-pSC23 plants to ~20% (Figure 4C). The reduction in the editing efficiency of the endogenous *ndhD* transcript in the transgenic plants indicates that increasing the demand for *ndhD* editing leads to the depletion of an editing factor which is present in limiting amounts. Interestingly, the decrease in editing efficiency was due to the presence of chimeric mRNA in quantities comparable to those of the endogenous *ndhD* mRNA (data not shown). However, the efficiency of editing of the *psbL* transcript in the transgenic Nt-pSC23 plants was the same as in the wild-type plants (>99%; Figure 4C). Since *psbL* editing in the Nt-pSC23 plants is unaffected, the depleted editing factor is *ndhD*-specific, and is not required for *psbL* editing.

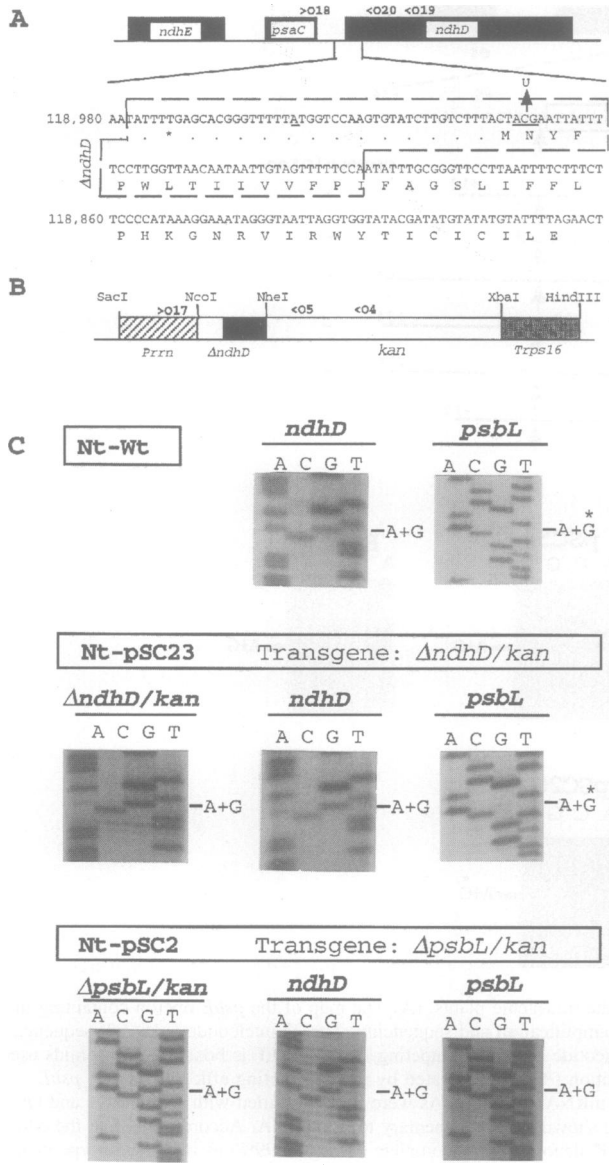

Fig. 4. Chimeric mRNAs containing the *ndhD* editing site do not compete for *psbL*-SEF. (**A**) Partial map of the tobacco plastid genome containing the *ndhD*, *psaC* and *ndhE* genes, and the DNA sequence with the edited *ndhD* translation initiation codon (underlined). The genes are marked and the DNA sequence is numbered according to Shinozaki *et al.* (1986). The Δ*ndhD* segment in a dashed box was translationally fused with the *kan* gene, as shown in Figure 4B. The position of primers O18, O19 and O20 are indicated. Note the A nucleotide 26 bp upstream of the editing site (underlined) which was changed to a C during construction of the chimeric gene. (**B**) The Δ*ndhD/kan* chimeric gene in plasmid pSC23 expressed in the P*rrn*/T*rps16* cassette. The positions of primers O4, O5 and O17 are indicated. (**C**) Editing of *ndhD* and *psbL* sites in wild-type (Nt-wt), Nt-pSC23 and Nt-pSC2 plants. Editing of the *ndhD* site was studied in the endogenous *ndhD*, and the chimeric Δ*ndhD/kan* mRNAs. Editing of the *psbL* site was studied in the endogenous *psbL*, and the chimeric Δ*psbL/kan* mRNAs. The *ndhD* cDNA was amplified with primers O18 and 019, and sequenced with primer O20. The *psbL* cDNA was PCR amplified with primers O1 and O6 and directly sequenced with primer O7. The Δ*ndhD/kan* and Δ*psbL/kan* cDNAs were amplified with primers O17 and O4, and sequenced with O5. Due to the polarity of the sequencing primers, the sequence shown is complementary to the mRNA. Accordingly, A at the edited position indicates a C to U conversion event, G an unedited C nucleotide.

We also tested *ndhD* editing in plants expressing the chimeric *kan* gene fused with the 98 nucleotide Δ*psbF*/Δ*psbL* fragment (Nt-pSC2, Figure 1). In such plants we have shown reduced editing of the endogenous *psbL* mRNA due to competition for *psbL*-SEF (Chaudhuri *et al.*, 1995; Figure 4C). However, in the same plants the endogenous *ndhD* editing is unaffected (Figure 4C) indicating that *psbL*-SEF is not involved in editing the *ndhD* site.

Discussion

We report here the first analysis of *cis*-element requirements for mRNA editing in plastids. We have found that C to U conversion in the *psbL* mRNA is directed by a 22 nucleotide sequence which encompasses 16 nucleotides upstream and five nucleotides downstream of the edited C at position 0. However, the entire 22 nucleotide sequence may not be required for editing, as sequences between −16/−10 and +5/+1 have not been tested. The 22 nucleotide sequence is conserved in tobacco, spinach (Kudla *et al.*, 1992) and bell pepper (Kuntz *et al.*, 1992), species in which editing of the *psbL* translation initiation codon has been reported.

The efficiency of editing of the initiation codons in the chimeric *psbL/kan* and *ndhD/kan* constructs is lower than those of the endogenous *psbL* and *ndhD* mRNAs. The reasons for this could be formation of RNA secondary or tertiary structures which decrease editing efficiency in the chimeric context, or the lack of sequences required for efficient editing. Alternatively, the higher fraction of unedited chimeric mRNAs could be due to faster RNA synthesis or RNA turnover rates.

The role of nucleotides directly flanking the editing site was tested by mutating them in the 98 nucleotide Δ*psbF*/Δ*psbL* fragment which is efficiently edited. Changing the upstream A at −1 to a C completely abolished editing of the correct C. However, changing the G at +1 to a C allowed editing of the correct C although at a reduced efficiency. Editing of the correct C in the mutated ACC codon indicates the high fidelity of nucleotide selection for editing. This is consistent with the observation that specific C nucleotides are edited within flanking C sequences (Kössel *et al.*, 1993; Maier *et al.*, 1995). Furthermore, editing of the ACC codon suggests that translation initiation is not required for editing to occur, providing direct evidence for the lack of linkage between translation and editing. This finding is consistent with mRNA editing in plastids lacking ribosomes (Zeltz *et al.*, 1993) and with editing of unspliced plastid mRNAs which are not translatable (Freyer *et al.*, 1993).

The *psbL* translation initiation codon is only one of the ~25 editing sites found in the plastids of higher plants (Maier *et al.*, 1995). Further studies will be required to determine how typical is the close proximity of *cis*-sequences to the editing sites in plastids that we have found for *psbL*. In this regard, the *ndhD* initiation codon appears to be similar, since all information required for editing is contained in a relatively small (89 nucleotides) RNA segment. Clustering of *cis*-sequences within a short segment is also true of some of the *ndhB* editing sites (Bock *et al.*, 1996). However, editing of sites II and III in the tobacco *ndhB* gene (Maier *et al.*, 1992) requires

sequences further away than 150 nucleotides (S.Chaudhuri and P.Maliga, unpublished). Therefore, localization of editing *cis* sequences is not uniform, in line with the proposed individual recognition mechanism for each of the ~25 plastid editing sites.

Individual recognition of the editing sites is consistent with the finding that site-specific *trans*-factors are depleted by overexpression of the *psbL* and *ndhD* target RNAs. While ACG to AUG editing in both transcripts creates a translation initiation codon, overexpression of either of the target RNAs affects the editing efficiency of only the source mRNA.

It is likely that C to U editing in plastids involves cytidine deamination, as shown for plant mitochondria (Yu and Schuster, 1995). Editing therefore minimally involves either a single polypeptide containing both a site-specific recognition domain and a deaminase domain, or a complex containing at least two components, one providing site-specific recognition and the other with cytidine deaminase activity. Such a multi-component complex consisting of cytidine deaminase (APOBEC-1) and auxiliary proteins has been shown to be involved in C to U editing of the mammalian nuclear apolipoprotein B mRNA. In addition to the common occurrence of C to U editing, close clustering of the *cis*-sequences around the editing site is an additional feature shared by the plastid *psbL* and the mammalian nuclear apolipoprotein B editing systems. Editing of apolipoprotein B is directed by an 11 nucleotide recognition sequence located four nucleotides downstream of the editing site. In addition, sequences upstream are required for efficient editing (reviewed in Innerarity *et al.*, 1996). However, in contrast to editing of *psbL*, recognition specificity of the apolipoprotein B editing process is relaxed, since Cs introduced adjacent to the edited nucleotide may also be modified (Chen *et al.*, 1990). Further experiments will clarify to what extent editing in plant organelles shares common features with editing of nuclear transcripts in mammals.

Testing of RNA editing from chimeric gene constructs *in vivo* is a significant step forward to dissect the process in plastids. However, the effort required to generate transplastomic lines limits the number of constructs which can be analyzed. This limitation can be overcome only through the development of an *in vitro* editing system for plastids.

Materials and methods

Construction of chimeric genes

The *psbL* deletion derivatives and the *ndhD* gene fragment were generated by PCR amplification with 5′ primers carrying *Nco*I restriction site and 3′ primers carrying *Nhe*I restriction site using total cellular DNA from tobacco (*Nicotiana tabacum* cv. Petit Havana). The following primer pairs were used: plasmid pSC2, O23 and O29; plasmid pSC3, O23 and O30; plasmid pSC4, O23 and O31; plasmid pSC5, O23 and O32; plasmid pSC6, O24 and O29; plasmid pSC7, O25 and O29; plasmid pSC8, O26 and O29; plasmid pSC9, O27 and O29; plasmid pSC10, O28 and O29; plasmid pSC18, O27 and O31; plasmid pSC19, O27 and O34; plasmid pSC20, O33 and O31; and plasmid pSC23, O37 and O38. The PCR products were digested with *Nco*I and *Nhe*I restriction enzymes.

The *psbL* derivatives with a point mutation were obtained by the megaprimer method of PCR (Sarkar and Sommer, 1990) using plasmid pSC2 as the template. These were also designed as *Nco*I and *Nhe*I fragments. The primers used were the following: plasmid pSC14, step I, O35 and O29, step II, O23; plasmid pSC15, step I, O36 and O29, step II, O23.

To introduce suitable restriction sites at the 5′-end of the *kan* coding region, *kan* was PCR amplified from pTNH32 (Carrer *et al.*, 1993) using 5′ primer (O21) carrying *Nco*I and *Nhe*I restriction sites in tandem and 3′ primer (O22) carrying *Xba*I restriction site. The PCR product was cloned in *Nco*I–*Xba*I digested pUC120 to generate plasmid pSC1.

The chimeric genes were constructed by N-terminal fusion of PCR amplified sequences from tobacco *psbL* and *ndhD* genes (*Nco*I–*Nhe*I fragments) to bacterial *kan* gene lacking the initiation codon (*Nhe*I–*Xba*I fragments). The chimeric genes were then cloned in *Nco*I–*Xba*I digested plasmid pLAA24A (Zoubenko *et al.*, 1994). Plasmid pLAA24 is a derivative of plastid transformation vector pPRV111A, which has a selectable spectinomycin resistance gene, and a *uidA* reporter gene in the P*rrn*/T*rps16* expression cassette (Zoubenko *et al.*, 1994). The P*rrn* 5′-regulatory region consists of the plastid rRNA operon promoter and a ribosome binding site and is on an *Eco*RI–*Nco*I fragment. The T*rps16* fragment includes the *rps16* gene 3′-regulatory region between nucleotides 5087–4939 in the ptDNA (Shinozaki *et al.*, 1986) and is contained within an *Xba*I–*Hin*dIII fragment. Digestion of plasmid pLAA24A with *Nco*I–*Xba*I restriction enzymes removes the *uidA* coding region from the expression cassette, which is then replaced with the chimeric constructs, also an *Nco*I–*Xba*I fragment.

Plastid transformation and plant regeneration

Tobacco (*N.tabacum* cv. Petit Havana) plants were grown aseptically on agar-solidified medium containing MS salts (Murashige and Skoog, 1962) and sucrose (30 g/l). Leaves were placed abaxial side up on RMOP media for bombardment. The RMOP medium consists of MS salts, N^6-benzyladenine (1 mg/l), 1-naphthalene acetic acid (0.1 mg/l), thiamine (1 mg/l), inositol (100 mg/l), agar (6 g/l) at pH 5.8 and sucrose (30 g/l). The DNA was introduced into chloroplasts on the surface of 1 μm tungsten particles using the Biorad PDS1000He Biolistic gun (Maliga, 1995). Spectinomycin resistant clones were selected on RMOP medium containing 500 mg/ml of spectinomycin dihydrochloride. Resistant shoots were regenerated on the same selective medium, and rooted on MS agar medium (Svab and Maliga, 1993).

cDNA synthesis, PCR amplification, DNA sequencing and PhosphorImager analysis

Total cellular RNA was extracted using TRIzol (Gibco BRL). Reverse transcription of proteinase K- and DNase I-treated RNA samples were carried out as described by Kudla *et al.* (1992). cDNA were amplified by PCR according to standard protocols: 1 min at 92°C, 2 min at 55°C, 1.5 min at 72°C, 30 cycles. Direct sequencing of PCR amplification products was carried out using the Sequenase PCR product sequencing kit (USB). The sequencing gels were subjected to PhosphorImager analysis (Molecular Dynamics) for quantitation of editing efficiency. Radioactivity in bands corresponding to nucleotides was determined. The values were normalized for sample loading and labeling efficiency against other bands in the same lanes. mRNA editing efficiency (%) = [corrected edited signal/(corrected edited + corrected unedited signal)]×100.

List of primers

O1: 5′-CAATATCAGCAATGCAGTTCATCC-3′
O4: 5′-CACGACGAGATCCTCGCCG-3′
O5: 5′-GAATAGCCTCTCCACCCA-3′
O6: 5′-GGAATCCTTCCAGTAGTATCGGCC-3′
O7: 5′-GGAAAATAAAACAGCAAGTAC-3′
O17: 5′-AATTCGAAGCGCTTGGATACAGTTGTAGGGA-3′
O18: 5′-GTAAGAGATGTGAATCCGCCTGT-3′
O19: 5′-GCATAAGTCGTTAGAAGGAG-3′
O20: 5′-GAAGAAAGAAAATTAAGGAACC-3′
O21: 5′-CATGCCATGGCTAGCATTGAACAAGATGGATTGCACG-3′
O22: 5′-GTACTCTAGACCCGCTCAGAAGAACTCG-3′
O23: 5′-CTAGCCATGGCTTTGGGATCAATATCAGCAATG-3′
O24: 5′-CTAGCCATGGCATCAGCAATGCAGTTCATCC-3′
O25: 5′-CTAGCCATGGCGTTCATCCAACGATAAACTTAA-3′
O26: 5′-CTAGCCATGGCATAAACTTAATCCGAATTATAGAG-3′
O27: 5′-CTAGCCATGGCCGAATTATAGAGCTACGACAC-3′
O28: 5′-CTAGCCATGGCTACGACACAATCAAACCCGA-3′
O29: 5′-CTAGCTAGCTTCAACATTTTGTTCGTTCGG-3′
O30: 5′-CTAGCTAGCTTCGTTCGGGTTTGATTGTG-3′
O31: 5′-CTAGCTAGCTGATTGTGTCGTAGCTCTATA-3′
O32: 5′-CTAGCTAGCCGTAGCTCTATAATTCGGATT-3′
O33: 5′-CTAGCCATGGTATAGAGCTACGACAC-3′
O34: 5′-CTAGCTAGCAAGTGTCGTAGCTCTATA-3′
O35: 5′-AATTATAGAGCTCCGACACAATC-3′

O36: 5′-AATTATAGAGCTACCACACAATC-3′
O37: 5′-CTAGCCATGGTATTTTGAGCACGGGTTTTTCTGGTCC-3′
O38: 5′-CTAGCTAGCTGGAAAAACTACAATTATTGTTAACC-3′

Acknowledgements

This research was supported by the National Science Foundation Grant MCB 93-05037 to P.M.

References

Araya,A., Domec,C., Begu,D. and Litvak,J. (1992) An *in vitro* system for editing of ATP synthase subunit 9 mRNA using wheat mitochondrial extracts. *Proc. Natl Acad. Sci. USA*, **89**, 1040–1044.

Benne,R. (1994) RNA editing in trypanosomes. *Eur. J. Biochem.*, **221**, 9–23.

Bock,R. and Maliga,P. (1995) *In vivo* testing of a tobacco plastid DNA segment for guide RNA function in *psbL* editing. *Mol. Gen. Genet.*, **247**, 439–443.

Bock,R., Kössel,H. and Maliga,P. (1994) Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype. *EMBO J.*, **13**, 4623–4628.

Bock,R., Hermann,M. and Kössel,H. (1996) *In vivo* dissection of *cis*-acting determinants for plastid RNA editing. *EMBO J.*, **15**, 5052–5059.

Bonnard,G., Gualberto,J.M., Lamattina,L. and Grienenberger,J.M. (1992) RNA editing in plant mitochondria. *Crit. Rev. Plant Sci.*, **10**, 503–524.

Carillo,N., Seyer,P., Tyagi,A. and Herrmann,R.G. (1986) Cytochrome b-559 genes from *Oenothera hookeri* and *Nicotiana tabacum* show a remarkably high degree of conservation as compared to spinach. The enigma of cytochrome b-559: Highly conserved genes and proteins but no known function. *Curr. Genet.*, **10**, 619–624.

Carrer,H., Hockenberry,T.N., Svab,Z. and Maliga,P. (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. *Mol. Gen. Genet.*, **241**, 49–56.

Chan,L. (1993) RNA editing: exploring one mode with apolipoprotein B mRNA. *BioEssays*, **15**, 33–41.

Chaudhuri,S., Carrer,H. and Maliga,M. (1995) Site-specific factor involved in the editing of the *psbL* mRNA in tobacco plastids. *EMBO J.*, **14**, 2951–2957.

Freyer,R., Hoch,B., Neckermann,K., Maier,R.M. and Kössel,H. (1993) RNA editing in maize chloroplasts is a processing step independent of splicing and cleavage to monocistronic mRNAs. *Plant J.*, **4**, 621–629.

Gray,M.W. and Covello,P.S. (1993) RNA editing in plant mitochondria and chloroplasts. *FASEB J.*, **7**, 64–71.

Hanson,M.R., Sutton,C.A. and Lu,B. (1996) Plant organelle gene expression: altered by RNA editing. *Trends Plant Sci.*, **1**, 57–64.

Innerarity,T.L., Boren,J., Yamanaka,S. and Olofsson,S.O. (1996) Biosynthesis of apolipoprotein B48-containing lipoproteins. *J. Biol. Chem.*, **271**, 2353–2356.

Kössel,H., Hoch,B., Maier,R.M., Igloi,G.L., Kudla,J., Zeltz,P., Freyer,R., Neckermann,K. and Ruf,S. (1993) RNA editing in chloroplasts. In Kueck,U. and Brennicke,A. (eds), *Plant Mitochondria*. VCH Publishers, Weinheim, Germany, pp. 93–102.

Kudla,J., Igloi,G.L., Metzlaff,M., Hagemann,R. and Kössel,H. (1992) RNA editing in tobacco chloroplasts leads to the formation of a translatable *psbL* messenger RNA by a C-to-U substitution within the initiation codon. *EMBO J.*, **11**, 1099–1103.

Kuntz,M., Camara,B., Weil,J.H. and Schantz,R. (1992) The *psbL* gene from bell pepper (*Capsicum annuum*): plastid RNA editing occurs in non-photosynthetic chromoplasts. *Plant. Mol. Biol.*, **20**, 1185–1188.

Maier,R.M., Neckermann,K., Hoch,B., Akhmedov,N.B. and Kössel,H (1992) Identification of editing positions in the ndhB transcript from maize chloroplasts reveals sequence similarities between editing sites of chloroplasts and plant mitochondria. *Nucleic Acids Res.*, **20**, 6189–6194.

Maier,R.M., Neckermann,K., Igloi,G.L. and Kössel,H. (1995) Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. *J. Mol. Biol.*, **251**, 614–628.

Maliga,P. (1995) Biolistic transformation of tobacco cells with nuclear drug resistance genes. In Maliga,P., Klessig,D., Cashmore,A., Gruissem,W. and Varner,J. (eds), *Methods in Plant Molecular Biology – A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 73–54.

Murashige,T. and Skoog,F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.*, **15**, 473–497.

Neckermann,K., Zeltz,P., Igloi,G.L., Kössel,H. and Maier, R.M. (1994) The role of RNA editing in conservation of start codons in chloroplast genomes. *Gene*, **146**, 177–182.

Sarkar,G. and Sommer,S.S. (1990) The 'megaprimer' method of site-directed mutagenesis. *Biotechniques*, **8**, 404–407.

Schuster,W. and Brennicke,A. (1994) The plant mitochondrial genome: physical structure, information content, RNA editing, and gene migration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 61–78.

Scott,J. (1995) A place in the world for RNA editing. *Cell*, **81**, 833–836.

Shinozaki,K *et al.* (1986) The complete nucleotide sequence of the tobacco chloroplast genome: Its gene organization and gene expression. *EMBO J.*, **5**, 2043–2049.

Simpson,L. and Thiemann,O.H. (1995) Sense from nonsense: RNA editing in mitochondria of kinetoplastid protozoa and slime molds. *Cell*, **81**, 837–840.

Sutton,C.A., Zoubenko,O.V., Hanson,M.R. and Maliga,P. (1995) A plant mitochondrial sequence transcribed in transgenic tobacco chloroplasts is not edited. *Mol. Cell. Biol.*, **15**, 1377–1381.

Svab,Z. and Maliga,P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. *Proc. Natl Acad. Sci. USA*, **90**, 913–917.

Yu,W. and Schuster,W. (1995) Evidence for a site-specific cytidine deamination reaction involved in C to U editing of plant mitochondria. *J. Biol. Chem.*, **270**, 18227–18233.

Zeltz,P., Hess,W.R., Neckermann,K., Boerner,T. and Kössel,H. (1993) Editing of the chloroplast *rpoB* transcript is independent of chloroplast translation and shows different patterns in barley and maize. *EMBO J.*, **12**, 4291–4296.

Zoubenko,O.V., Allison,L.A., Svab,Z. and Maliga,P. (1994) Efficient targeting of foreign genes into the tobacco plastid genome. *Nucleic Acids Res.*, **22**, 3819–3824.

Received on April 30, 1996; revised on July 1, 1996