Translation of tobacco chloroplast *rps14* mRNA depends on a Shine-Dalgarno-like sequence in the 5′-untranslated region but not on internal RNA editing in the coding region

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Received 8 May 1998

Abstract The role of Shine-Dalgarno-like sequences in mRNAs from higher plant chloroplasts has not been analyzed experimentally so far. In vitro translation analysis has revealed that the Shine-Dalgarno-like sequence is essential for translation of tobacco chloroplast *rps14* mRNA. Two RNA editing sites have been identified in the protein-coding region of the *rps14* mRNA. Editing of the second site was found to be partial and hence the partially edited transcripts are accumulated in tobacco green leaves. In vitro translation assays using the fully edited, partially edited and unedited *rps14* mRNAs indicated that editing does not directly influence translational efficiency.

Key words: Chloroplast; Shine-Dalgarno sequence; RNA editing; Tobacco; Translation

1. Introduction

Translational initiation and its efficiency are determined by specific sequence elements and RNA secondary structures in mRNAs. *Cis*-acting translational elements are present in the 5′-untranslated regions (UTRs), 3′-UTRs and/or even protein-coding regions. Shine-Dalgarno (SD) sequences which are commonly found 7 ± 2 nt upstream from initiation codons in the 5′-UTR of *Escherichia coli* mRNAs are critical for ribosome binding and accurate translational initiation [1]. In higher plant chloroplasts, many genes encoding polypeptides possess upstream sequences similar to the bacterial SD sequence. Spacing of these chloroplast SD-like sequences is less conserved, ranging from −2 to −29 with respect to initiation codons [2]. However, no experimental evidence has been reported for the exact role of SD-like sequences in translation in higher plant chloroplasts. In the green alga *Chlamydomonas reinhardtii*, mutation analysis of chloroplast SD-like sequences has yielded conflicting results for the importance of this sequence on translational initiation in vivo. Deletion of the SD-like sequence from its *psbA* 5′-UTR abolished translation [3], while replacement mutagenesis of the SD-like sequence in the 5′-UTRs of its *petD* had no effect on translation [4]. Replacement mutagenesis of SD-like sequences located in the −149 to −19 regions of four *Chlamydomonas* chloroplast genes had no effect on translation [5]. We previously reported that an SD-like sequence (GGAG) in the 5′-UTR of tobacco *psbA* mRNA has no influence on translation and that two other sequences in the 5′-UTR, termed RBS1 and RBS2, both of which are complementary to the 3′-end of tobacco 16S rRNA, are critical elements for translational initiation [6].

RNA editing was found as a novel post-transcriptional event in higher plant chloroplasts, first identified in maize *rpl2* [7] and tobacco *psbL* transcripts [8]. Subsequently, RNA editing was found in various transcripts from chloroplasts in all major lineages of land plants [9–12]. All of the chloroplast RNA editing found so far in angiosperms and gymnosperms is C-to-U conversions that lead mostly to amino acid substitutions to restore conserved peptide sequences in other species, suggesting that residues acquired by editing are important for the encoded protein function. Actually, the introduction of a spinach editing site into the tobacco plastid genome showed that the heterologous site remains unmodified due probably to the lack of a species-specific factor(s) and that the lack of RNA editing leads to a mutant phenotype [13]. A limited amount of editing occurs in the third nucleotide of codons (silent editing) [14] and untranslated regions [15], which does not cause amino acid substitutions. In the hornwort *Anthoceros formosae*, U-to-C inverse editing was found in addition to C-to-U editing [10,11]. Chloroplast RNA editing was initially reported to be entirely independent of chloroplast translation [16], however, recently Karcher and Bock reported that elevated temperature and inhibition of chloroplast translation by antibiotics selectively block RNA editing at a small number of sites, suggesting a role of the chloroplast translation in RNA editing [17].

In plant mitochondria, many C-to-U editing sites (and rarely U-to-C) have been found [18,19]. Although unedited transcripts of some plant mitochondrial genes are extremely low in abundance, transcripts of many other genes are less homogeneous; partially edited transcripts are readily detected and often are more abundant than transcripts that are fully edited [20]. Amino acid sequence analysis of the ATP synthase subunit 9 from potato mitochondria demonstrated that the isolated subunit reflects the fully edited translation product, even though the corresponding mRNA population exhibits incomplete editing [21]. In addition, single homogeneous forms of ATP6 and NAD9 polypeptides were reported to be accumulated, despite the presence of incompletely edited mRNAs of the corresponding genes [22,23]. On the other hand, incomplete editing of *rps12* transcripts was shown to result in the synthesis of polymorphic polypeptides in maize mitochondria [24].

The *rps14* mRNA from tobacco chloroplasts possesses an SD-like sequence in the 5′-UTR [25]. Here we show that two new RNA editing events occur in the protein-coding region of *rps14* mRNA, and that editing of one site is partial, resulting in the accumulation of the partially edited *rps14* mRNA. Using our chloroplast in vitro translation system, we present

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PII: S0014-5793(98)00673-5

experimental evidence that the SD-like sequence is essential for translation while the edited and unedited mRNAs are equally translated.

2. Materials and methods

Tobacco (*Nicotiana tabacum* BY4) leaves were harvested from 4-week-old plants in a growth chamber at 28°C under 16 h light/8 h dark conditions. Pea leaves were harvested from 2-week-old plants grown as above. Chloroplast RNA and DNA from tobacco and pea leaves were prepared as described [14]. cDNA synthesis was carried out according to the instruction manual of the cDNA cycle kit (Invitrogen) using random primers and 1 μg DNase I-treated RNA as template. cDNA and chloroplast DNA fragments were amplified by polymerase chain reaction (PCR) as described [14], purified by 1% agarose gel electrophoresis and sequenced using the DNA cycle sequencing kit (Stratagene). Tobacco *rps14* gene (positions −149 to +260) [25], with or without mutations (see Figs. 1A and 3A), were amplified from the tobacco DNA or cDNA by PCR and cloned into pBluescript II SK$^+$ to construct *rps14-lacZ* fusion genes or native *rps14* genes. Plasmid DNAs were linearized with *Bgl*II, extracted with phenol/chloroform and precipitated with ethanol. mRNA templates were synthesized using T3 Megascript (Ambion). Preparation of tobacco chloroplast extracts and in vitro translation reaction were carried out as described [6].

3. Results and discussion

3.1. An SD-like sequence is essential for translation of tobacco chloroplast rps14 mRNA

The *rps14* gene encoding the ribosomal protein S14 (100 amino acids) is cotranscribed with the *psaA-psaB* genes encoding the P700 apoproteins of photosystem I (Fig. 1A) [25]. In order to analyze translation in vitro of the *rps14* mRNA, the authentic coding region (the first 86 codons) was extended by fusing a *lacZ* portion (79 codons) so as to facilitate detection of in vitro translation product (19 kDa) by PAGE (WT in Fig. 1A). In vitro translation analysis revealed that this *rps14-lacZ* mRNA is accurately translated to produce a polypeptide of the expected size of 19 kDa (Fig. 1B, lane 2). In its 5′-UTR, an SD-like sequence (GGA) is located at the −14 position, although this position is far from the conserved position of SD sequences observed in *E. coli* (−5 to −9). To investigate the function of this SD-like sequence, a mutant *rps14* mRNA with CCU instead of GGA (M-SD in Fig. 1A) was translated in vitro. As shown in Fig. 1B (lane 3), translation was almost completely abolished by this mutation, indicating that the SD-like sequence, GGA, is essential for translation of *rps14* mRNA even though its position is relatively far from the initiation codon as compared with that of *E. coli*. In higher plant chloroplasts, the spacing between the SD-like sequence and the initiation codon is highly variable [2] and no direct evidence indicating the importance of SD-like sequences has been reported. We previously showed that two separate sequences AAG and UGAU (RBS1 and RBS2, respectively), both of which are not similar to the typical SD sequence, contribute to the translational initiation of *psbA* mRNA in tobacco chloroplasts, while a typical SD-like sequence (GGAG, RBS3) located at −37 was not important [6]. Our in vitro experiment provides the first experimental evidence that the SD-like sequence is required for translation of some mRNAs in higher plant chloroplasts. Therefore, the distance between SD-like sequences and initiation codons is suggested to be important for chloroplast ribosomes to recognize the proper initiation codon.

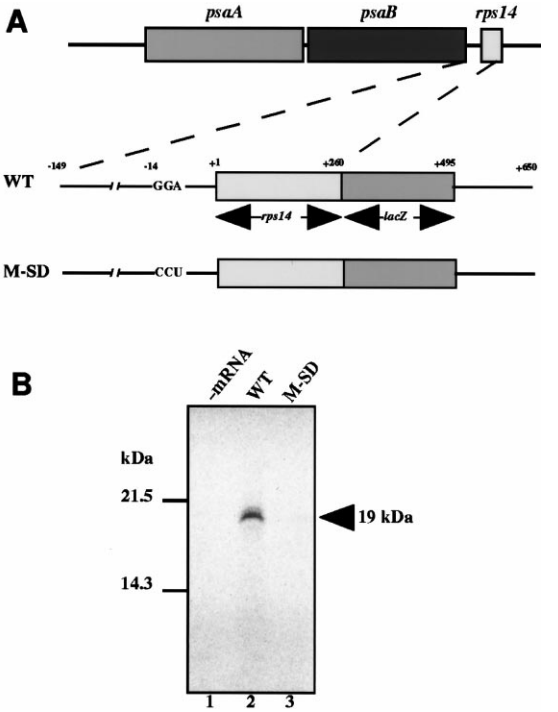

Fig. 1. Translation in vitro of wild type and mutant chloroplast *rps14* mRNAs. A: Schematic representation of the *psaA-psaB-rps14* cluster [25] and mRNA templates. Wild type (WT) and the mutant (M-SD) *rps14-lacZ* fusion mRNAs are shown below. Nucleotide positions are from its start codon (A is +1). B: Each mRNA template was incubated for 30 min at 30°C with the tobacco in vitro translation system (25 μl). ^{35}S-labeled products were separated by PAGE and visualized by Bioimaging analyzer BAS2000. Size markers are trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa).

3.2. RNA editing of rps14 transcripts in tobacco and pea chloroplasts

According to the amino acid alignment of ribosomal protein S14 sequences, two potential RNA editing sites in which conserved amino acid residues are restored by C-to-U conversions were noticed in the coding region of tobacco *rps14* gene (Fig. 2C). Direct sequencing of PCR amplified cDNA fragments revealed that both sites (I and II) were edited. UCA (Ser) at the 27th codon (nt position +80) and CCA (Pro) at the 50th codon (nt position +149) are converted to UUA (Leu) and CUA (Leu), respectively (Fig. 2A). Both of the acquired leucine residues contribute to the conservation of S14 proteins among most chloroplasts and *E. coli*, suggesting that these leucine residues are critical for the protein function.

As can be seen in Fig. 2B, site I is fully edited while site II is partially edited. Based on the band intensity of sequencing ladders, approximately 40% of the *rps14* mRNA pool in tobacco green leaves are accumulated as partially edited mRNA, probably as an editing intermediate. Sequence analysis of cloned *rps14* cDNAs revealed that two clones were fully edited at both sites while other two clones were partially edited, only at site I, and site II remained unedited (data not shown). Partial RNA editing in chloroplasts has been reported at some editing sites in spinach *psbF* and *psbL* mRNAs [26], *ndhD* mRNAs of three angiosperm species [27], tobacco *atpA* mRNAs [14], and black pine *petL*(*ycf7*)-*petG* mRNAs [9]. The extent of partial editing often changes depending on developmental and environmental conditions [14,17,26,28],

suggesting that change of editing extent, at least for ACG to AUG conversion, is one of the determinants of the synthesized amount of functional proteins or of the translational efficiency [28].

With respect to site I, editing was identified in maize [29] and is expected in spinach, the parasitic plant *Epifagus virginiana*, pea and rice. Pea has the proline codon (CCA) at site I while UCA (Ser) in tobacco. Analysis of the cDNA sequence of pea *rps14* transcripts revealed that editing takes place at site I (Fig. 2C). As for site II, it is expected in spinach, *Epifagus* and a gymnosperm, black pine. Black pine has the serine codon (UCA) at site II, instead of the proline codon (CCA) in tobacco. We found that the serine codon (UCA) is edited to produce a leucine codon (UUA) in black pine chloroplasts (Fig. 2C) [9]. Hence, editing at site I and site II leads to the conserved leucine codon from different amino acid codons. Our data suggest that the recognition of these sites by the editing machinery has changed during evolution. An additional possibility is that the immediately upstream nucleotide (the first nucleotide of codons, C or U) is not critical for the recognition of the editing machinery.

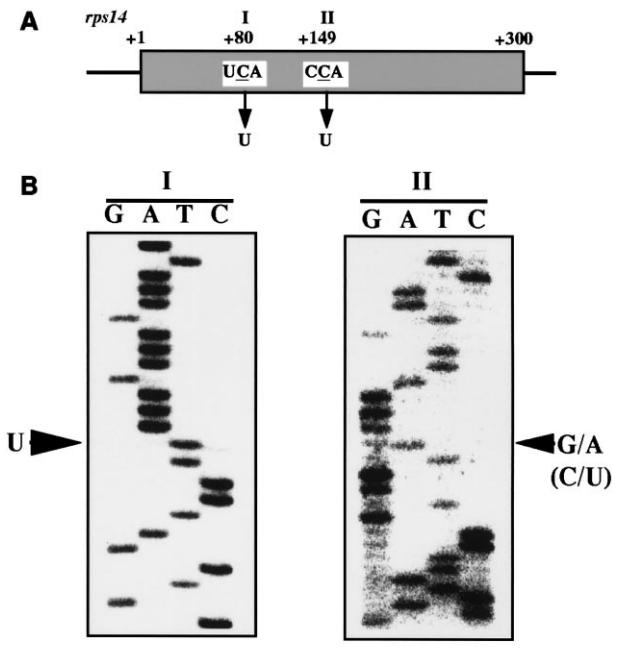

Fig. 2. RNA editing in transcripts from the chloroplast *rps14* gene. A: Schematic representation of the *rps14* gene. Nucleotide positions are from its start codon (A is +1). C at position +80 (site I) and C at position +149 are converted to U. B: Direct sequencing of PCR-amplified cDNAs containing two RNA editing sites (I and II). The site II sequence was analyzed on the complementary strand (A/G corresponds to U/C in mRNA). C: Amino acid alignment of S14 protein portions including sites I and II. Gaps (asterisks) are introduced to align sequences. Circled residues are converted to leucines (L) by editing. Tobacco and pea are from this article, maize from [29] and black pine from [9].

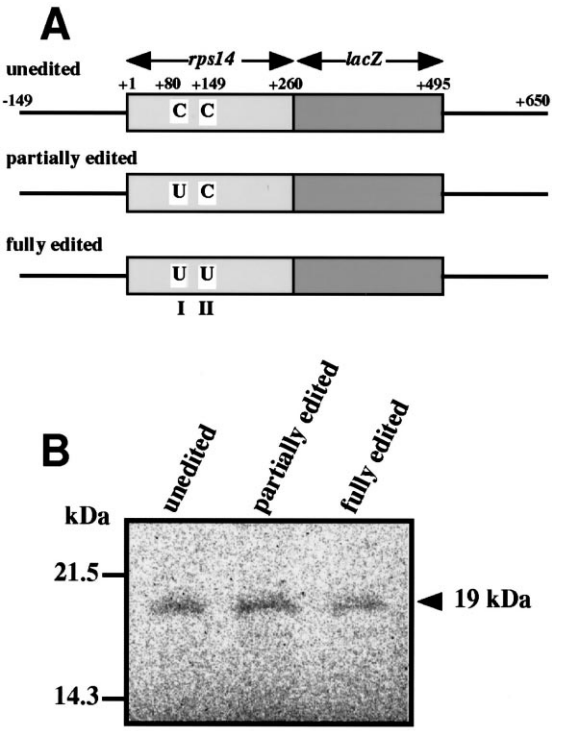

Fig. 3. Translation in vitro of unedited, partially edited and fully edited *rps14* mRNAs. A: Schematic representation of three mRNA templates. B: Synthesized products (19 kDa) from each mRNA were separated by PAGE.

3.3. *In vitro translation of fully edited, partially edited and unedited chloroplast rps14 transcripts*

It is now known that translation is sometimes affected not only by elements in untranslated regions but also by those in coding regions [1]. In order to dissect the influence of RNA editing within the protein-coding region on translation, three kinds of *rps14-lacZ* mRNA templates, each of which is differentially edited at site I and site II (Fig. 3A), were constructed. In vitro translation assay revealed that the amount of expected translation product (19 kDa) from each mRNA is similar, suggesting that editing in the protein-coding region does not affect translation efficiency of *rps14* mRNAs. This was confirmed by in vitro translation using fully edited, partially edited and unedited authentic *rps14* mRNAs, not fused to *lacZ*. These three mRNAs were translated to produce a 9 kDa polypeptide (corresponding to the chloroplast ribosomal protein S14) (data not shown). The possibility that the unedited *rps14* mRNAs were edited during in vitro translation reaction could be ruled out, because no editing was observed by sequence analysis of the cDNAs synthesized from ribosome-associated mRNAs in this reaction (data not shown).

No experimental evidence indicating the in vivo accumulation of polypeptides translated from unedited or partially edited mRNAs has been reported in chloroplasts. As reported in plant mitochondria, the aberrant polypeptides synthesized from incompletely edited mRNAs are likely to be non-functional or harmful because the amino acid residues substituted by editing are thought to be important for protein function [24]. The importance of the leucine residue in the *petB* protein, which is restored by editing in maize and tobacco [30,31], was

recently shown in *Chlamydomonas* chloroplasts [32]. Our present study suggests that editing within a protein-coding region does not affect the translation efficiency in chloroplasts, probably due to the fact that no structured change of mRNA is caused by the editing, and hence aberrant polypeptides are synthesized from partially edited and unedited mRNAs. The selective degradation of aberrant polypeptides may be an additional post-translational step important for the determination of functional protein levels.

Acknowledgements: We thank Drs. M. Sugita and T. Wakasugi for their support and useful discussions, Dr. T. Tsudzuki for computer analysis. This work was supported in part by a Grant-in-Aid from the Ministry of Education (Japan).

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