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Internal Editing of the Maize Chloroplast *ndhA* Transcript Restores Codons for Conserved Amino Acids

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The NADH dehydrogenase subunit A (*ndhA*) gene from maize chloroplasts encodes a highly conserved peptide, which at several positions could be restored to consensus sequences by potential C-to-U editing of the codons involved. This gene was, therefore, chosen for analysis of its mRNA sequence in the form of amplified cDNA. A comparison of this cDNA sequence with the plastome-encoded *ndhA* sequence reveals four C-to-U editing sites, thereby demonstrating as a novel finding that chloroplast editing can also affect internal mRNA positions. All the edited codons restore amino acids that are conserved in the *ndhA*-encoded peptides of other chloroplast species. Alignment with homologous mitochondrial NADH-ubiquinone reductase subunit 1 (*nad1*) sequences of plant and even nonplant species shows that two of the editing positions restore universally conserved amino acids and that one editing site is even shared with *nad1* mRNA of plant mitochondria. No editing sites could be detected in the cDNA derived from transcripts of the maize chloroplast RNA polymerase α-subunit (*rpoA*) gene.

INTRODUCTION

Editing of mRNA was first described for transcripts encoded in kinetoplast DNA (Benne, 1990; Stuart, 1991). In this case, insertions and deletions of U residues directed by guide RNAs (Blum et al., 1991) may lead to substantial divergence between nucleotide sequences of mature mRNAs and their respective genes. Subsequently, editing was also found for several nuclear and mitochondrial mRNAs. In contrast to kinetoplast editing, sequence alteration in the latter case is caused by base substitution or deamination (Weissmann et al., 1990; Mulligan, 1991). Plant mitochondria mRNAs, in particular, are extensively edited, usually by C-to-U (and occasionally U-to-C) transitions (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989).

Editing in chloroplasts has only recently been detected for two mRNAs in which AUG initiator codons are created from ACG triplets by C-to-U conversions (Hoch et al., 1991; Kudla et al., 1992). The question therefore arises as to whether chloroplast editing is restricted to the rare cases where nonfunctional initiator codons are encoded in their respective genes or whether editing also occurs at a higher frequency within internal codons of chloroplast mRNAs, as is the case in plant mitochondrial mRNAs. To address this question, the genes coding for subunit A of NADH dehydrogenase (*ndhA*) and for the α-subunit of RNA polymerase (*rpoA*) from maize chloroplasts have been selected for analysis at both the genomic DNA and the cDNA levels. These two genes are well-conserved representatives of the known higher plant plastomes (Ohyama et al., 1986; Shinozaki et al., 1986; Hiratsuka et al., 1989) and have also been shown to produce abundant transcript levels (Matsubayashi et al., 1987; Ruf and Kössel, 1988; Vera et al., 1990). It was therefore anticipated that analysis of their cDNAs would reveal editing sites, if editing in chloroplast plays a major role in gene expression by also involving internal mRNA positions. In particular, an alignment of the *ndhA*-encoded peptides lent support to this expectation because it showed several positions where amino acid consensus sequences could be restored by C-to-U transitions within the respective codons.

Although the *rpoA* gene does not show such potential editing sites at strongly conserved positions, it was also included in this investigation for the following reasons. Overexpression of the *rpoA*-encoded peptide, which we are currently studying with a genomic clone in an *Escherichia coli* expression system, would generate a mutant peptide if editing took place in the chloroplast system. Therefore, to ensure production of a wild-type peptide, an absence of editing had to be proven. In addition to this, a search for *rpoA* editing sites was thought to yield further information on the frequency of chloroplast editing sites in general and/or on their occurrence in codons for nonconserved amino acids and in silent positions.

Here, we present experimental evidence for the existence of four C-to-U editing sites within the *ndhA*-encoded transcript of maize chloroplasts, which, as a novel finding, shows that editing can also occur at internal sites and at several positions of a chloroplast mRNA. In all four cases, editing restores codons for conserved amino acid positions of the *ndhA*-encoded peptide. Two of the restored positions are universally conserved in the homologous NADH-ubiquinone reductase subunit 1

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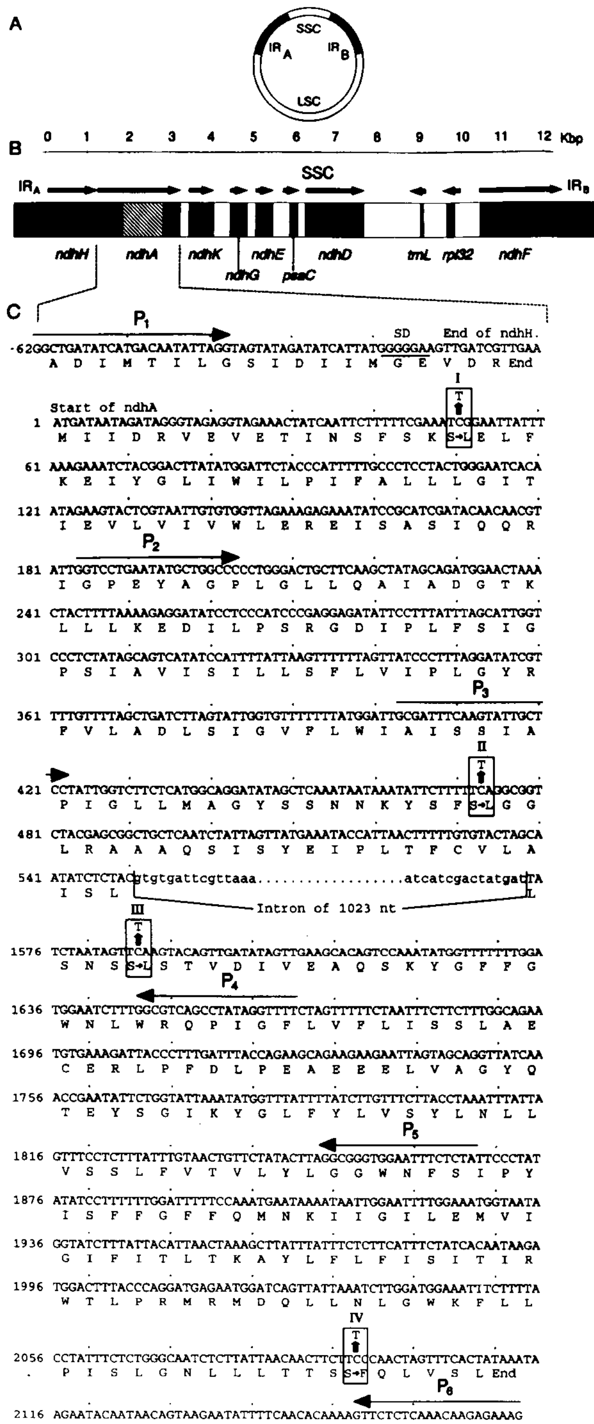

Figure 1. Position and Structure of the *ndhA* Gene of the Maize Plastome.

(A) Location of the *ndhA* gene within the small single-copy region (SSC), in relation to the two inverted repeats IR_A and IR_B, and the large single-copy region (LSC) of the maize plastome.

(B) Position of the *ndhA* gene within the *ndh* gene cluster and other genes of the small single-copy region. The intron of the *ndhA* gene is marked by striations. The direction of transcription is indicated by arrows.

(C) Nucleotide sequence of the two *ndhA* exons including the intron boundary sites. The positions and orientations of the primers P_1 (5′ primer) and P_6 (3′ primer) used for the polymerase chain reaction and sequencing, and of the additional sequencing primers P_2, P_3, P_4, and P_5, are indicated by horizontal arrows. A potential ribosomal binding site proximal to the *ndhA* gene is marked by underlining. The four editing positions I to IV where C-to-U transitions alter the amino acid sequence are boxed.

(*nad1*) genes of mitochondria. The plant mitochondrial *nad1* gene is also the target of editing at one of these sites, suggesting that the editing machinery of both organellar systems may share common components. In contrast to the editing sites observed for *ndhA*-encoded mRNA, no editing could be detected for *rpoA*-encoded mRNA.

RESULTS

Identification of a Chloroplast-Specific cDNA Derived from a *ndhA*-Encoded Transcript

As depicted in Figures 1A and 1B, the *ndhA* gene is part of the *ndh* gene cluster residing in the small single-copy region of the plastome of higher plants (Ohyama et al., 1986; Shinozaki et al., 1986; Hiratsuka et al., 1989). The peptides encoded by this and other chloroplast *ndh* genes are homologous to subunits of mitochondrial NADH-ubiquinone reductase (Matsubayashi et al., 1987; Fearnley et al., 1989) that are encoded by mitochondrial or nuclear *nad* genes. This has led to the postulation of a respiratory chain located in chloroplasts (i.e., chlororespiration; for a review, see Scherer, 1990). Transcripts of chloroplast *ndh* genes are abundant in tobacco (Matsubayashi et al., 1987; Lin and Wu, 1990), barley (Vera et al., 1990), and spinach (Lin and Wu, 1990). The genes may, therefore, specify subunits of a chloroplast NADH dehydrogenase, although no protein product encoded by the chloroplast *ndhA* gene has been described so far. In Figure 1C, the sequence of the two *ndhA* exons from maize together with the positions and orientations of the primers P_1 (5′ primer) and P_6 (3′ primer) used for amplification and sequencing are shown. Also depicted are the additional sequencing primers P_2, P_3, P_4, and P_5. It should be noted that the sequence of primer P_1 is derived from the proximal *ndhH* gene that in maize is separated from the *ndhA* gene by only a single base pair (Maier et al., 1990), whereas primer P_6 is complementary to a sequence of the *ndhA*/*ndhK* intergenic region.

Amplification of either maize chloroplast DNA or chloroplast cDNA derived from maize chloroplast RNA with the primer pair P_1P_6 leads to products of the expected lengths of 2235 and 1212 bp, respectively, as shown in Figure 2, lanes 2 and 3. The shorter length of the cDNA amplification product reflects the loss of the 1023-nucleotide intron, thereby ensuring that

the cDNA is derived from a processed *ndhA* transcript. Formation of the cDNA amplification product suggests also that the *ndhA* coding transcript is physically linked with the *ndhH* transcript, and it appears therefore likely that the entire *ndh* gene cluster constitutes a single multicistronic transcription unit. The possibility, however, that the *ndhA* and *ndhH* transcripts are separate units and that primer P_1 merely acts from a 5' leader region of the *ndhA* transcript extending into the *ndhH* gene cannot be ruled out.

In view of the gene transfer from the plastome to mitochondrial DNA that has been observed in several cases (Stern and Lonsdale, 1982; Lonsdale et al., 1983; Stern and Palmer, 1986), the possibility of an amplification product derived from a transferred *ndhA* gene had to be excluded by control amplifications with a primer pair specific for the mitochondrial subunit 2 of the cytochrome oxidase (*cox2*) gene (Fox and Leaver, 1981; Hoch et al., 1991; Pruitt and Hanson, 1991). This yields a product corresponding to the expected length of 514 bp with mitochondrial cDNA (lane 6 of Figure 2); it failed, however, to produce an amplification product with chloroplast cDNA (lane 5), which demonstrates the absence of contaminating mitochondrial sequences in the chloroplast cDNA preparation and therefore excludes a mitochondrial origin of the 1212-bp amplification product observed in lane 3 of Figure 2.

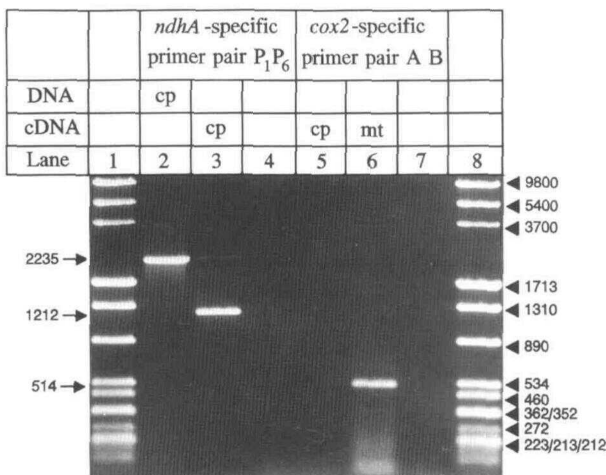

Figure 2. Amplification Products Obtained by Polymerase Chain Reaction.

Primer combination P_1P_6 that is specific for the maize chloroplast *ndhA* gene and primer pair AB that is specific for the mitochondrial *cox2* gene from maize were used in lanes 2 to 4 and 5 to 7, respectively. Amplifications were carried out either with DNA from maize chloroplasts (lane 2), or with cDNA obtained by reverse transcription of RNA from maize chloroplasts (lanes 3 and 5) or maize mitochondria (lane 6). The positions and expected lengths in base pairs of the amplification products are marked by horizontal arrows at left; the position of the length markers (lanes 1 and 8) are indicated by arrowheads at right. Control reactions using buffer instead of DNA were run in lanes 4 and 7.

Several Editing Sites Occur at Internal Positions of *ndhA*-mRNA

The amplification products from plastome DNA and cDNA were directly used for sequence analysis of the complete *ndhA* gene and of the coding region of the *ndhA* transcript, respectively. Sequence analysis of the plastome *ndhA* sequence derived from the 2235-bp amplification product obtained in lane 2 of Figure 2 and from the plasmid pZmc404 of a maize chloroplast DNA clone bank (Maier et al., 1990; data not shown) was conducted. For sequence analysis of the *ndhA* cDNA, the 1212-bp product obtained from lane 3 of Figure 2 was used. This allowed identification of four positions in which the cDNA sequence deviates from the plastome sequence by C-to-U transitions. Sequence autoradiograms showing the four editing positions and their surrounding regions are depicted in Figure 3 (cf. the respective positions within the *ndhA* sequence given in Figure 1C). Whereas complete C-to-U transitions are observed for the editing positions I to III (Figures 3A to 3C), a certain amount of unedited cDNA at position IV (Figure 3D) can be recognized. It remains to be seen whether the isoform of the peptide encoded by the mRNA unedited at this position is translated in vivo and can be incorporated into a functional NADH dehydrogenase complex or whether this apparent slower rate of editing is compensated by other processes.

The proximity of editing positions II and III to the intron/exon boundaries also allows exact localization of the latter at the position where the cDNA sequences diverge from the plastome DNA sequence (Figures 3B and 3C). The intron/exon boundaries thus determined for the maize *ndhA* gene are in accordance with the boundaries identified for the tobacco *ndhA* gene (Matsubayashi et al., 1987).

Editing Results in Restoration of Codons for Conserved Amino Acids

An alignment of amino acid sequences encoded by the mRNA sequences surrounding the four maize editing sites with homologous sequences of chloroplast *ndhA* gene products and of mitochondrial *nad1* gene products is depicted in Figure 4. At site I, for which homologous sequences are only present in the chloroplast peptides, editing creates a UUG leucine codon (see also Figure 1C) that corresponds to the leucine residue encoded in the plastome DNA of the three other species. Thus, editing results in the conservation of this apparently functionally important amino acid residue. The difference between the two monocotyledons maize and rice, with the leucine residue of the latter being encoded at the gene level, shows that editing positions may diverge even between closely related species.

The UUA leucine codons (see also Figure 1C) created at editing sites II and III again restore the highly conserved leucine residues observed in other *ndhA* genes and in most of the mitochondrial *nad1* genes. It appears likely, therefore,

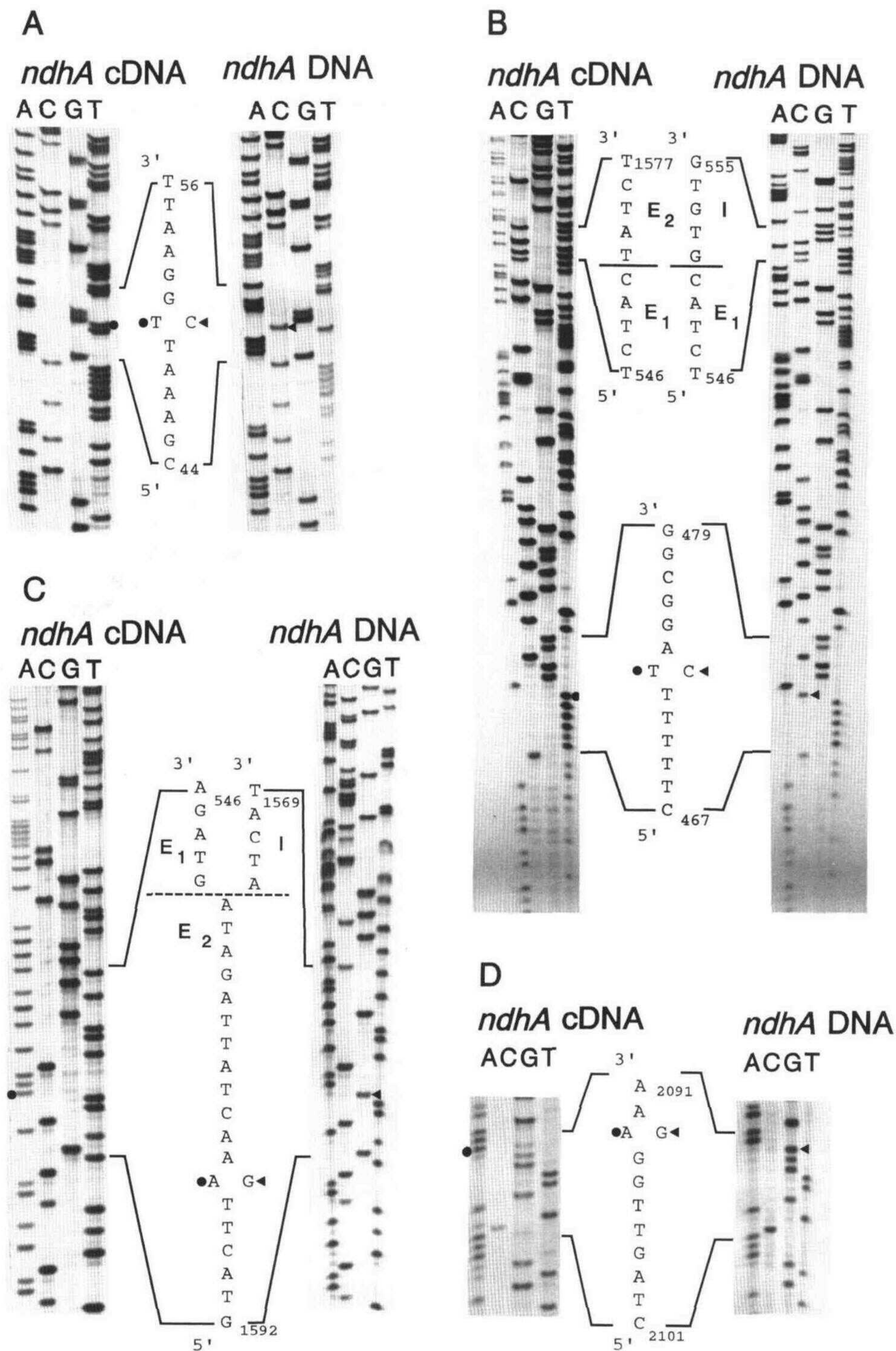

Figure 3. Identification of Editing Positions in the *ndhA* Transcript by Comparison of the *ndhA* Nucleotide Sequences Obtained with either Amplified cDNA or Amplified Plastome DNA as Templates.

The sequencing autoradiograms show the following regions of the *ndhA* DNA or cDNA sequence.

(A) Editing site I.

(B) Editing site II and 5′ exon/intron border (E_1/I) or 5′ exon/3′ exon border (E_1/E_2).

(C) Editing site III and 3′ exon/intron border (I/E_2) or 5′ exon/3′ exon border (E_1/E_2).

(D) Editing site IV.

The editing positions, where chloroplast DNA and cDNA differ, are marked by arrowheads (DNA) and solid circles (cDNA). Because of the polarity of the primers P_4 and P_6, the autoradiograms in **(C)** and **(D)** show sequences complementary to the *ndhA* mRNA. Sequences around the intron/exon borders in **(B)** and **(C)** are marked by E_1 (first exon), E_2 (second exon), and I (intron).

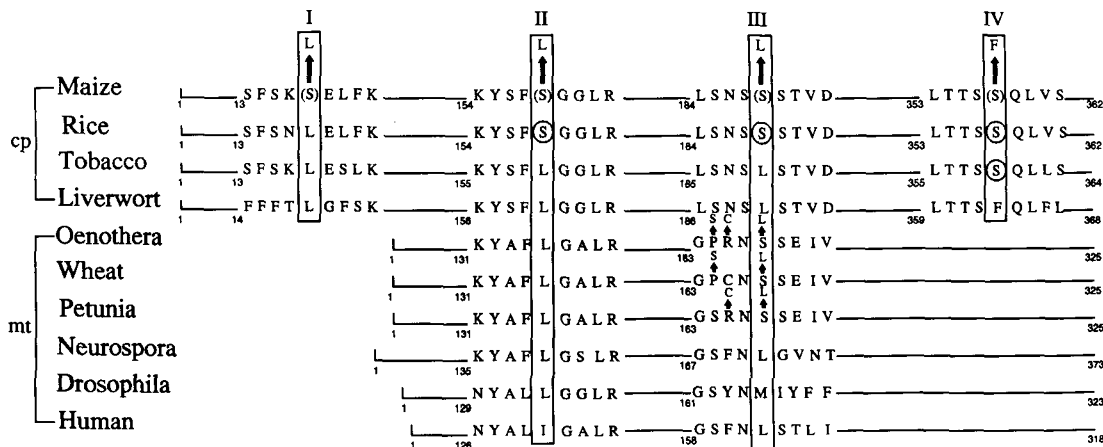

Figure 4. Restoration of Amino Acids in the Maize *ndhA*-Encoded Peptide.

The amino acid sequence encoded by the maize *ndhA* regions containing the four editing positions I to IV is aligned with homologous sequences from rice (Hiratsuka et al., 1989), tobacco (Shinozaki et al., 1986), and liverwort (Ohyama et al., 1986). Amino acid sequences encoded in the homologous regions of the mitochondrial *nad1* genes of Oenothera (Wissinger et al., 1991), wheat (Chapdelaine and Bonen, 1991), petunia (Conklin et al., 1991), Neurospora (Burger, 1985), Drosophila (Clary and Wolstenholme, 1985), and humans (Anderson et al., 1981) are also included. Amino acid substitutions resulting from editing are marked by small vertical arrows. Circling indicates amino acid positions in rice and tobacco that are most likely to be restored to conserved amino acid residues by editing of the respective codons.

that the UCA serine codons found at sites II and III of rice (Hiratsuka et al., 1989) are also converted to UUA leucine codons by editing. The same holds for the UCC serine codon of site IV in rice and tobacco that is probably edited to the UUC phenylalanine codon, as it is in maize. With respect to the liverwort sequence, it is noteworthy that all the amino acid residues restored by editing in maize (and inferred to result from editing in rice and tobacco) are conserved by the respective codons existing at the gene level. It appears likely, therefore, that editing in the chloroplasts of the phylogenetically distant liverwort is minimal or perhaps even nonexistent. In summary, the codon changes created at the four editing sites restore conserved amino acid residues that are probably essential for the function of the peptides encoded by the *ndhA* genes.

Chloroplast *ndhA* and Plant Mitochondrial *nad1* Share a Common Editing Site

As shown in Figure 4, the serine-to-leucine transition produced at the editing site III of the chloroplast *ndhA* gene is observed at the exactly homologous position of the *nad1* genes from plant mitochondria (Chapdelaine and Bonen, 1991; Conklin et al., 1991; Wissinger et al., 1991), whereas conservation of the leucine residue in the other mitochondrial *nad1* genes, including the human homolog, is effected by the existence of leucine codons at the gene level. This conservation of an editing site between chloroplasts and plant mitochondria raises the possibility that the editing process in both the plant organelles may share common mechanistic steps and/or some of the necessary components. On the other hand, two plant mitochondrial editing sites in proximity to site III—leading to two proline/serine and two arginine/cysteine transitions—are not maintained in the chloroplast *ndhA* genes. The two proline-to-serine transitions, however, again restore a universally conserved serine residue (also conserved but not edited in the chloroplast sequences), whereas the cysteine residues resulting from the two arginine-to-cysteine transitions are in concordance with only the plant mitochondrial family.

rpoA mRNA Is Not Edited

To obtain a more general picture of the frequency of chloroplast editing, we are currently analyzing several other chloroplast mRNAs by cDNA sequencing. We have conducted a complete analysis of the *rpoA* gene that encodes the α-subunit of chloroplast RNA polymerase (Ruf and Kössel, 1988) and that is located at the distal end of the large cluster of ribosomal proteins (Hoch et al., 1991). Surprisingly, no sequence deviations between the *rpoA* gene and the *rpoA* cDNA could be detected. This absence of editing in the *rpoA* mRNA shows that the distribution of editing sites in chloroplasts is very uneven with respect to individual mRNAs and probably rather moderate in general, especially when compared with the high frequency observed in plant mitochondria.

DISCUSSION

The identification of several editing sites in the *ndhA* transcript from maize chloroplasts demonstrates that editing of chloroplast mRNAs is not restricted to the rare cases in which ACG

triplets have to be converted to functional AUG initiator codons (Hoch et al., 1991; Kudla et al., 1992). The current findings also extend chloroplast editing previously noted for a ribosomal protein gene (Hoch et al., 1991) and a photosynthetic gene (Kudla et al., 1992) to a member of a third gene class, the *ndh* genes, thereby demonstrating that this novel step of chloroplast gene expression is probably not limited to certain gene classes. It even appears conceivable that editing occurs within intron sequences or rRNA transcripts, as has been observed in plant mitochondria (Schuster et al., 1991; Wissinger et al., 1991).

The creation of initiator codons observed for the chloroplast editing sites identified earlier appears indicative of a regulatory role for the editing process whereby conversion of nonfunctional to translatable mRNAs is achieved. On the other hand, the main function of the editing events described here is the restoration of codons for conserved amino acids, thereby ensuring functionality of the encoded peptide. This view is strengthened by the absence of neutral editing sites, which, as occasionally observed for the editing of plant mitochondrial mRNAs, would lead to either identical codons or to codons causing only neutral amino acid substitutions. It remains to be seen whether a higher specificity of the chloroplast editing process, at least with respect to its biological function, can be inferred by identification of other chloroplast editing sites; this is suggested from the few sites identified so far and from the absence of editing sites in the *rpoA* transcript.

Nothing is known about the mechanism of the editing process and its enzymatic apparatus. Progress in this direction will depend on the development of an in vitro editing system. A comparison of sequences surrounding the four chloroplast editing sites described here and of the two editing sites described earlier (Hoch et al., 1991; Kudla et al., 1992) has so far not revealed any consensus sequences that might function as an editing signal. Also, the codons per se are unlikely to function as editing signals because identical codons occur in the *ndhA* mRNA in edited and nonedited positions, e.g., the TCG codon of editing site I that is preceded by the same codon only two codons upstream (Figure 1C). The possibility that the additional information necessary for the editing process may reside in distant parts of the plastome has yet to be considered. Further testing is required to determine whether this information is mediated by guide RNAs analogous to the editing mechanism acting in kinetoplasts (Blum et al., 1991).

METHODS

Isolation of Organelles

Intact chloroplasts were isolated from 10-day-old green maize seedlings by Percoll gradient centrifugation as described by Robinson and Barnett (1988). Mitochondria were isolated from 6-day-old etiolated maize coleoptiles by differential centrifugation and sucrose gradient centrifugation according to Slater (1991).

Nucleic Acid Preparation

Nucleic acids from purified chloroplasts and mitochondria were prepared by guanidinium-hydrochloride and CsCl gradient centrifugation (Chirgwin et al., 1979). The RNAs were then DNase I treated, phenol-chloroform extracted, and precipitated with 2 volumes of ethanol in the presence of 0.3 M NaOAc, pH 4.8. After CsCl gradient centrifugation, chloroplast and mitochondrial DNAs were recovered from the supernatants of the pelleted RNAs by five extractions with NaCl-saturated isopropanol. After the addition of 2 volumes of water to the lower phase, the DNAs were precipitated with 1 volume of isopropanol.

Reverse Transcription

RNA was reverse transcribed with a random primer mixture using avian myoblastosis virus reverse transcriptase. Twenty micrograms of DNase I treated RNA and 1 nmol of a hexanucleotide random primer mixture were incubated for 5 min at 60°C in the presence of 40 mM morpholinopropanesulfonic acid, pH 6.7, 400 mM NaCl, and 1 mM EDTA. For primer annealing, the reaction mixture was cooled to 30°C over a period of 30 min and immediately precipitated with 2.5 volumes of ethanol in the presence of 0.3 M NaOAc, pH 4.8. To initiate cDNA synthesis, the redissolved pellet was incubated for 3 hr at 42°C in the presence of 50 mM Tris, pH 8.3, 10 mM $MgCl_2$, 50 mM KCl, 3 mM DTT, 1 mM deoxynucleoside triphosphate, 54 units of RNA guard (Pharmacia LKB Biotechnology Inc.), and 25 units of avian myoblastosis virus reverse transcriptase (Angewandte Gentechnologie Systeme GmbH, Heidelberg, Germany). The reaction was terminated by addition of 0.2 volumes of 0.5 M EDTA, pH 8. To hydrolize the RNA, the product mixture was ethanol precipitated, and the pellet was incubated in 0.3 M NaOH, 5 mM EDTA, pH 8, for 30 min at 65°C. The sample was neutralized by the addition of 1.2 volumes of 1 M Tris, pH 7.5, and precipitated with 2.5 volumes of ethanol.

Amplification of DNA and cDNA by Polymerase Chain Reaction

DNA and cDNA were amplified according to a standard protocol with 32 cycles of 93°C (for 30 sec), 55°C (for 1 min), and 72°C (for 1 min) with a 2-min extension at 93°C of the first cycle and a 7-min extension at 72°C of the final cycle in the presence of 1.5 mM magnesium chloride. Amplification products were separated on 1.5% agarose gels and eluted onto a DEAE membrane.

Sequencing

Gel-purified amplification products were sequenced directly by a modified chain termination method described by Bachmann et al. (1990). Plasmids were sequenced by the dideoxy chain termination method using fluorescent primers for labeling of the products and analyzed as described by Hoch et al. (1991).

List of Oligonucleotides

Oligonucleotides used for the polymerase chain reaction and sequencing were synthesized on a DNA synthesizer (model No. 394; Applied Biosystems, Foster City, CA).

The positions of primers P_1 to P_6 are shown in Figure 1C. The positions of the primers A and B specific for subunit 2 of the cytochrome oxidase (*cox2*) gene were described previously (Hoch et al., 1991).

P_1: 5'-GGCTGATATCATGACAATATTAGG-3'
P_2: 5'-GGTCCTGAATATGCTGGCCC-3'
P_3: 5'-GCGATTTCAAGTATTGCTCC-3'
P_4: 5'-AAAACCTATAGGCTGACGCC-3'
P_5: 5'-ATAGAGAAATTCCACCCGCC-3'
P_6: 5'-CTTTCTCTTGTTTGAGAGAAC-3'
A: 5'-GCTGCGGAACCATGGCAATTAG-3'
B: 5'-GAGGTACATCAGCGGGTGTTAC-3'

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